Lymphocyte Proliferation In Vitro Alteration by Benzo[a]pyrene, Diesel Exhaust Extract and Sodium Arsenite

By

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ABSTRACT

Biological actions in humans typically result in a range of measurable responses. Some of the difference or variation in biological responses (inter-individual variation) is due to factors such as diet, age, genetics, smoking, exercise, lifestyle and health status. Previous or ongoing chemical exposures can also contribute to variation. The current study focuses on evaluating inter-individual differences in the background proliferative capacity of human T-lymphocytes and in the ability of T-lymphocytes to respond to three environmentally relevant toxicants; benzo[a]pyrene (B(a)P), diesel exhaust extract (DEE) and sodium arsenite (SA). Any alteration in an individual's lymphocyte proliferative response resulting from xenobiotic exposure could change immunocompetance.

The goals of this study were 1) to evaluate in vitro proliferation of T-lymphocytes in response to three toxicants. We observed a greater inhibition of cellular proliferation in T-lymphocytes treated with As than with B(a)P or DEE. For all three toxicants statistically significant differences from untreated controls were seen at the highest dose only. 2) To characterize inter-individual differences of baseline proliferative capacity based on age or gender. Pediatric subjects were not statistically different from adults. Men had a higher proliferative response than women. 3) To determine if baseline proliferative capacity was predictive of individual responses. Differences in baseline levels of proliferative capacity were predictive of the extent to which the individual responded to the toxicants. 4) To determine the utility of this experimental design for use in field epidemiological studies. This study design and the lymphocyte proliferation

assay can provide useful biomarker data; baseline data need to be established for the study subjects, and several repeat samples should be analyzed.

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1. Introduction

Human exposure to environmental pollutants has been recognized to induce adverse effects on the immune system. However, the extent of these adverse immune effects is still unclear. In order to evaluate the extent of these effects several factors must first be considered. First, it is necessary to demonstrate that a toxicant (environmental pollutant) induces immunotoxic effects, and secondly, it is important to characterize the variability of these adverse effects in a population. In our study we have evaluated the dose-responsiveness, and comparative potency of three environmentally-relevant chemicals (benzo[a]pyrene, B(a)P; diesel exhaust extract, DEE; and sodium arsenite, (SA) utilizing an in vitro assay to quantitate changes in cellular proliferation of stimulated human lymphocytes, which are an integral cellular component of the immune system. Also, due to the heterogeneity of the human population we have asked the question, can an individual's intrinsic proliferative capacity (stimulated lymphocytes) be predictive of subsequent enhancement or suppressive effects following toxicant exposure? Research information of this nature regarding the dose-responsiveness, comparative potency, and inter-individual variability of biological responses of the immune system will provide a better characterized assay for use in epidemiological field studies and may enable risk assessors to reduce uncertainty in human health risk assessments.

Risk Characterization

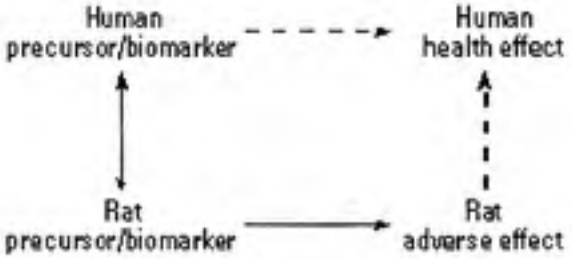
In 1983 the National Research Council published a manual focusing on the process of human health risk assessments and identified four primary disciplines; 1) hazard identification 2) dose-response assessment 3) exposure assessment, and 4) risk characterization (NRC, 1983). Briefly, hazard identification determines whether an environmental pollutant is directly associated with increased adverse health effect. Hazard identification is used to characterize the nature and strength of the association. Dose-response assessment focuses on the quantitative relationship between an exposure (dose) and the severity of the resulting adverse health effect. Quantitative exposure assessment evaluates the distinct characteristics and level of exposure to a pollutant, including the chemical nature of the pollutant, routes, whether exposure actually occurred and time course of exposure. Risk characterization takes into account the three previously mentioned assessments resulting in determination of possibility of harm to an exposed individual. Typically the dose-response assessment has a high degree of uncertainty due to inter-individual variability and species extrapolation.

A biological response is measured in individuals, the difference or variation in response defines the inter-individual variation. Many factors can contribute to this variation. These variations can be affected by diet, age, genetics, smoking, exercise, lifestyle, health status. Previous or current exposures to chemicals can contribute significantly to variation in biological responses following exposure to environmental pollutants (Perera, 1997; Iyaniwura, 2004). Variation in these factors, have been suggested to account for as much as 10 - 30 fold differences in biological responses (Caserez, 2001). Because substantial inter-individual variation exists, characterization of

this variation, and identification of which biological responses are affected will have significant value in delineating factors that can reduce the amount of uncertainty in human health assessments.

Species extrapolation is a process by which results of toxicity testing for environmental pollutants conducted in laboratory animals are applied to humans. Likewise, species extrapolation significantly contributes to uncertainty of a dose-response assessment. Laboratory animal studies provide an avenue to predict human toxicity to the same exposure, but it is important to realize that these animal models have both quantitative and qualitative limitations that contribute to the uncertainty.

To reduce the amount of uncertainty associated with the dose response assessment, a multi-pronged approach has been utilized (Sobels, 1982).



Shown here the approach utilizes a common biological endpoint by comparing data derived from human in vivo and in vitro (observed precursor/ biomarker) effects and observed laboratory animal in vivo (precursor/ biomarker) and in vitro (adverse effect) studies. Extrapolation between species offer significant benefits compared to the limited approach of conducting only human or only laboratory animal studies. Human studies are limited by the time needed to observe the effect and confounding issues (diet, exercise, health status, exposure to multiple pollutants, etc.) inadequate dose characterization, co-exposure to multiple pollutants, limitations on the nature and amount

of biological samples that can be collected, and the inability of obtaining tissue that may be most relevant to the biological response of interest. Similarly the utility of laboratory animal studies is limited by inter-species differences, non-relevant doses and time of exposure differences, when compared to actual human exposure scenarios, and limited genetic variation. Thus this multi-pronged approach capitalizes on the strengths of both human and laboratory animal studies by establishing consistency of effect between the human and animal in vitro and in vivo studies. The relationships presented by the multi-pronged approach provide increased predictive value for human adverse health effects by reducing the uncertainty associated with inter-species (species extrapolation) and intra-species (comparisons of biological responses; inter-individual variation) variability.

Biomarkers

An important component of the multi-pronged approach is the evaluation of a biomarker, which measures a biological response related to an adverse health effect in humans or a critical effect in laboratory animals. Biomarkers, as defined in the US National Academy of Sciences report (NRC, 1989b) include "any measurement reflecting an interaction between a biological system and a potential hazard, which may be functional and physiological, biochemical at the cellular level or a molecular interaction". The importance of developing and utilizing biomarkers is emphasized by organizations such as United Nations Environment Programme, World Health Organization International Labour Organisation and National Center for Toxicological Research, all actively engaged in biomarker research (WHO, 1993).

Biomarkers are usually divided into three classes, biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility. Biomarkers of exposure measure an individual's exposure to a xenobiotic (environmental pollutant) including determinations of a concentration of the xenobiotics or metabolites. Biomarkers of effect measure changes that occur due to an exposure to a xenobiotic, and can be qualitative or quantitative in nature. Biomarkers of effect often are more proximal to a health effect than biomarkers of exposure (NRC, 1992). Biomarkers of exposure can measure a xenobiotic substance, its metabolite or the product of metabolic interaction with a particularly sensitive molecule or cell. For example, Symanski et al (2001) used urinary levels of mandelic acid (MA) and phenylglyoxylic acid (PGA) as biomarkers of exposure to styrene in workers at plastics plants. In humans, styrene is oxidized by cytochrome P-450, hydrolyzed, and subsequently oxidized to form MA and PGA.

Rössner et al (2004) studying workers at two Czech nuclear power plants saw an alteration of metabolic process, by measuring a tumor suppressor protein, p53. Utilizing this as a biomarker of effect, workers at one plant had double the amount of p53 in their lymphocytes compared to other plant workers and controls.

The majority of known biomarkers of susceptibility are biomarkers of genetic susceptibility. Biomarkers of genetic susceptibility are usually evaluated by case-control epidemiological studies looking at polymorphic variants in cancer patients compared to the general population. Susceptibility can also be defined in terms of developing a biological response to an environmental exposure (Albertini, 2000). Evans et al (2003) reviewed literature to illustrate the importance of determining efficacy and toxicity of medications in humans. Evaluating numerous studies looking at several drug

metabolizing enzymes as biomarkers of susceptibility, the review demonstrates the high degree of variability within and between individuals.

Immune Function

The focus of the current study is on individual differences in immune function following toxicant exposure. Any alteration in normal lymphocyte proliferative response, caused by xenobiotic exposure, is considered an alteration to immunocompetence. This alteration can lead to immunosupression, immunostimulation, hypersensitivity or autoimmunity. Immunosupression, a generalized reduction in immunity, can increase susceptibility to infection or development of tumors. Alteration of immunocompetence can be seen as injury to the immune system (i.e. immunotoxicity) and is possibly precursory to a diseased state (Hodgson, 2001). Different biomarkers of effect, as measured by impact on cell populations, have been investigated to better understand the changes in the immune system (Soto, 2003; Li, 1998). The immune system is composed of two systems: innate immunity and adaptive immunity. Innate immunity is the first line of defense, often eliminating the insult before significant damage occurs. There is no immunologic memory associated with innate immunity therefore the second and third exposures result in the same response as the first. An example of innate immunity would be inflammation in response to an injury to the skin, which signals release of fluids and swelling to isolate the area from further contact with underlying tissue.

Adaptive immunity, the second line of defense, usually involves an antigen which frequently causes a specific response within the system it targets. This response causes

the generation of antibodies that can bind or signal an immunologic cascade of events. Adaptive immunity can further be broken down into cell-mediated (thymic) and humoral. Thymic (T) and humoral (B) lymphocytes, specific cells within the broad category of white blood cells thought to consist of monocytes, neutrophils, eosinophils, basophils and lymphocytes circulate throughout the body within the circulatory and lymphatic system. In a normal adult, 20-40% of circulating lymphocytes are T cells. Response against an antigen that involves lymphocytes is cell-mediated, specifically by the T-lymphocytes. Stobo et al. (1973) demonstrated the existence of distinctive subsets of T-lymphocytes, which are differentiated by a specific cell surface protein. This protein determines how the T cell will perform its cellular immune functions. This protein and its density on T-lymphocyte surfaces determine stimulatory indices when exposed to a proliferative agent. The study delineated that T-lymphocytes with high phytohemmaglutinin (PHA) response (high amounts of protein) were the amplifiers (which clonally expand the population of antigen specific cells through proliferation) and those with low response (low amounts) were the effectors [cytotoxic lymphocytes also called Natural Killer Cells (NK)]. Antigenic stimulation of the T-lymphocytes within the peripheral blood system consists of clonal amplification followed by generation of effector cells (Wu, 2000). This amplification involves growth and proliferation of the T-lymphocytes. This information provides the basis for choosing PHA as the proliferative agent of choice. The current study utilized this proliferative response or its subsequent suppression as a measure of alteration of immune function. We looked specifically at a biomarker of exposure, T-lymphocytes in individuals, stimulated with PHA and their variable response to subsequent treatment with three environmental agents.

Numerous studies have been conducted to better understand inter-individual variability in immune response and reduce uncertainty and its relationship to immunologic function resulting in populations with increased susceptibility. CD2, CD4, CD8 and CD 19, for example, are clusters of determination (CD), which are specific markers on T-lymphocytes that denote the cell function. Robinson et al. (1996) characterized the normal ranges of T-lymphocytes with these CD markers in children to contrast with known adult ranges. This study is important to pediatricians treating children with immune function disorders because the use of adult ranges to assess immune function / immunological diseases in children could make treatment more or less effective. In this study, 233 children ages 5-13 years were characterized based upon their total numbers of each of the specific CD markers of interest. These particular clusters were chosen because the authors believe they are representative of the main T-lymphocyte with these markers. This provides data on total number of T-cells and a good indication on Natural Killer cells in the form of CD2+. CD4+ is a measure of T-dependent immune responses. CD8+ elucidates which cells have cytotoxic or suppressive capabilities and CD19+ helps immune responses in cells of the B-cell lineage. Robinson et al. (1996) found that as children age, there is an increase in number and percentage of CD4+, and an increase in percentage of CD8+ but no noticeable change in number. CD19+ showed decreases in both the percentage and number of cells as the child ages. These changes, as a child ages, could greatly affect treatment of childhood immune disorders. Utilization of these data, in place of normally used adult cell numbers and percentages, would greatly aid treatment of children with immunological problems.

Physiological changes that occur in aging populations can also drastically affect an immune response. For example, as a person ages the thymus shrinks and by age sixty is virtually non-performing. The thymus is the site of T cell differentiation and a critical organ for the development of the body's immune system (Leslie, 2004). Woodhouse et al (1992) looking at implications related to drug therapy in the elderly, reviewed numerous studies and concluded that hepatic drug metabolism in older adults is decreased, affecting toxicant metabolism. Also affecting the metabolism of immunotoxicants, overall liver volume decreases, which directly reduces CYP450 levels.

Lymphocyte Proliferation in Vitro

Change in lymphocyte proliferation capacity is used to assess environmental pollutants and characterize susceptible individuals. Soto et al (2003) looked at proliferative responses of lymphocytes in 80 children exposed to arsenic via drinking water. Responses to stimulation were reduced in the exposed population, indicating a reduced immune function when chronically exposed to arsenic. Bocchieri et al (1995) utilized the lymphocyte proliferation assay to evaluate the status of asymptomatic HIV seropositive and control subjects. Significantly lower proliferative responses were seen in the seropositive compared to controls, indicative of reduced immune function in the HIV subjects. Using the lymphocyte proliferation assay, Li et al (1998) studied the long term effect of retroviral treatment of subjects with advanced HIV-1 infection. Holland et al. (2002) treated isolated lymphocytes from twelve pesticide applicators and a control group of non applicators, with 2,4-D and looked at the proliferative alterations. 2,4-D a commonly used pesticide, has been associated with non-Hodgkin's lymphoma. Looking

specifically at the replication index (RI) which measures lymphocyte proliferation, the pesticide applicators had a significantly higher RI than controls when treated over a three month period.

Immunosupression or stimulation, hypersensitivity or autoimmunity can occur when an individual is exposed to immunologic challenge (Descotes, 2004). Chang et al (1995) studied non-insulin dependent diabetics and their reduced lymphocyte proliferative responses when challenged with a mitogen. Thirty four diabetic subjects were tested to determine the cause of this proliferative response suppression. The study concluded that decreased expression of interleukin-2 receptor (IL-2R), on activated lymphocytes might be responsible for the decreased proliferation. This abnormality, i.e. the suppression in the immune system, is especially important in increased occurrence of infections. Coles et al (1998) illustrated the use of anti-lymphocyte antibodies CD 52 which reduces proliferation and causes prolonged T cell depletion, to suppress a proliferative response in multiple sclerosis (MS) patients. MS is an autoimmune disorder, where elevated levels of T cells attack the nerve myelin and secrete chemicals that can permanently disable nerve fibers. They used humanized monoclonal antibody Campath-1H in vitro, which is directed towards CD 52, to suppress lymphocyte proliferative responses. The study showed that when treated with a mitogen (PHA), proliferative responses normally elevated in MS patients were reduced. The study also showed a rise in B cell populations and a mononuclear cell population shift towards a T-helper cell (Th1) phenotype. This treatment showed a modulation of the immune response in MS patients whose normal state is inflammatory in nature.

To better identify the effect of alteration of immunocompetence, Luster (1988) developed a testing battery to assess chemical-induced immunotoxicity. The effort initiated by the National Toxicology Program at National Institute of Environmental Health Sciences, working in conjunction with several research organizations, looked at comparative sensitivity and reproducibility of immunological assays. The battery of assays was chosen because they were recognized by immunologists as valid for diagnosis of cell and humoral mediated immunity and host resistance. With these assays in mind and adding other consideration factors such as simplicity and reproducibility without loss of sensitivity, a two tier screening approach was chosen. Tier I screen included; hematology, complete blood counts and differentials; body and organ weights; cellularity and histology of the spleen thymus and lymph nodes; humoral-mediated immunity by IgM with sheep red blood cells (SRBC); cell-mediated immunity by lymphocyte blastogenesis to mitogen stimulation; and natural killer cell activity before and after toxicant exposure. Tier II tests were employed if functional changes were seen in Tier I. The mitogen stimulated lymphocyte blastogenesis cell immunity assay utilized in Tier I is commonly called the lymphocyte proliferation assay (LPA). The LPA, an in vitro biomarker assay, involves the stimulation of lymphocytes by a mitogen to induce proliferation and subsequent treatment with an immunotoxicant. The LPA alteration is considered as an indicator of immune system function (Luster, 1988). To stimulate the proliferation of lymphocytes, use of mitogens is necessary; several mitogens have been utilized for this stimulation. The recommended mitogen for T-lymphocyte proliferation is PHA (Nowell, 1960). When T- lymphocytes are treated with immunotoxicants, the resulting proliferation is either stimulated or suppressed and can be quantified. The

lymphocytes from peripheral circulating blood are isolated, stimulated, treated, cultured for three days and analyzed. The original methodology incorporated a radioactive nucleotide ([^{3}H]-thymidine) into the proliferating cell and measured the incorporation of radioactivity with a scintillation counter. Utilizing tetrazolium salt, MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) Mosmann (1983) developed a quantitative colorimetric assay. The tetrazolium ring is cleaved and reduced to a blue formazan and metabolized by mitochondrial dehydrogenase in viable lymphocytes. This method used MTT, a strict control based stimulation measure utilizing individuals responses as their own control, instead of the [^{3}H]-thymidine counts from a radioisotope as a control. Utilizing BALB/c mouse spleen cells stimulated with *Salmonella typhosa* lipopolysaccharide, the traditional [^{3}H]-thymidine technique and the new MTT incorporation method were compared. This study showed that only viable cells cleave the MTT which is the colorimetric feature for quantitation, that MTT response was linear for a wide range of cell numbers present (200-50,000) with absorbance measured directly proportional to the amount of living cells present. Green et al (1984) supplemented the tetrazolium salt development and showed effects on lymphocytes when treated with cytotoxic lymphokines, cytotoxic T-cells lines and tumor cells. Denizot et al (1986), utilizing stimulated murine spleen cells, eliminated the organic solvent in the MTT method which greatly increases the solubility of the formazan product, preventing the subsequent protein precipitation responsible for the high variability in the background. Gerlier et al (1986) showed MTT could be utilized for determination of mitochondrial activity and its application for assessing cytotoxicity of some drugs. The method was further enhanced by the development of a more soluble and stable tetrazolium compound

containing sulfonate groups (Cory, 1991). The MTT method was shown to be a useful alternative to the radioisotope method for quantitation and appreciated for its simplicity. Marshall (1995) completed an extensive review comparing the tritiated thimidine, the original tetrazolium (MTT) and the sulfonated derivative (MTS) in lymphocyte assays, concluding that the MTS was a useful, reproducible and quantitative assay. The color intensity is directly related to the enzymatic activity of the cells. This method was found to be accurate, reproducible, safe, economical and useful for as few as 3000 cells (Hussain 1993). Gieni et al (1995) found that low levels of cytokine, insufficient to induce proliferation (DNA synthesis and [^{3}H]-thymidine uptake) but sufficient levels to maintain cell viability could metabolize the MTT and MTS to colored formazan compounds. The cytokines and their cellular mitochondrial activity, even without proliferation allowed for detection by the subsequent conversion of the MTT and MTS to the quantifiable formazan dye, even with no induction of proliferation. This cytokine production of IL-2 and IL-4, can be particularly low, but using the tetrazolium salts, the study showed a higher precision and a lower standard deviation than the [^{3}H]-thymidine. Utilizing this LPA methodology as an endpoint for screening new drugs, Green et al (2002) studied T lymphocyte proliferation in exercising, well-trained male runners. The LPA with tetrazolium dye showed a biphasic response, with elevated levels during and immediately after exercise, and decreased levels after a recovery period compared to baseline. Zolnai et al (1998) combined the original version of MTS (CellTiter 96™) with [phenazine methosulfate (PMS)], an exogenous electron carrier and compared it to the [^{3}H]-thymidine studying proliferation of bovine lymphocytes and found the incubation period could be altered to improve signal/background ratios. This evolved into new

combined MTS and PMS as the MTT (CellTiter 96™); (Promega, WI) utilized in the current study.

Environmental Pollutants

Many environmental pollutants can affect cell mediated immunity in vitro and in vivo. Of interest in this study are benzo[a]pyrene, diesel exhaust extract (DEE) with multiple polycyclic-aromatic hydrocarbons (PAH) and sodium arsenite.

Benzo[a]pyrene (B(a)P), a PAH, is a by-product of incomplete combustion of organic substances. B(a)P and other hydrocarbons are emitted into the environment from many sources. Coal, fuel oil and diesel fuel combustion are the primary source of outdoor aromatic hydrocarbons. Indoors, cigarette smoke and cooking are the primary sources of the pollutant. Generally the B(a)P is associated with particulate matter, a by-product of the inefficiency of a combustion reaction (Grimmer, 1983). Aromatic hydrocarbons have little inherent biological activity but when metabolized form reactive metabolites, many of which are recognized as probable human carcinogens. In addition to carcinogenic effects PAHs have been observed to have immunological effects. Davila et al, (1996) found that BaP suppressed human blood peripheral T cell mitogenesis. BaP and 3-methylcholanthrene were found to be the most toxic of nine PAHs tested as measured by proliferative capacity. Treatment with alpha-napthoflavone (a known Ah receptor antagonist and inhibitor of CYP-450) blocked the suppressive effects of B(a)P. Burchiel et al (2001) found that with oral or subcutaneous treatment, suppression of humoral and cell mediated immunity occurred but also noted that at low exposure levels B(a)P may actually augment cell signaling pathways resulting in immune function

function enhancement. Ward et al (1986) found that immunosupression produced by 7, 12-dimethyl benz[a]anthracene (DMBA), another PAH, lasted 4-8 weeks days after the exposure to doses that resulted in metastasis of neoplastic cells. The study showed that immunosuppression can be observed at doses below toxic levels and that PAHs may produce tolerance. Most importantly, Ward et al found a correlation between immunosuppression by PAHs and decreased resistance to infectious agents or transplanted tumor cells in adult B6C3F1 mice. Laupeze et al (2001) looked at differentiation, maturation and function of monocyte derived dendritic cells. These cells play an important role in the development of immune response. Their data (Laupeze et al) showed that exposure to PAHs inhibited in vitro functional differentiation and maturation of the dendritic cells.

Diesel exhaust is a complex mixture of volatile organic compounds, particulate matter, nitrated polycyclic aromatic hydrocarbons, sulfur and nitrogen dioxides, formaldehyde, acrolein and sulfuric acid. The National Institute of Standards and Technology offers a diesel exhaust particle extract which is available as a standard reference material, (SRM 1975). While this extract (DEE) lacks any measurable B(a)P, it does contain at least 23 PAH and 18 nitro-substituted PAH (average molecular weight 285 g/mol). Diesel engines are used to power ships, machinery, locomotives and transport trucks. The emissions from these sources, in the form of exhaust, are of sufficient quantities to impact air quality. Because of difficulty in obtaining samples and measuring immunological effects are varied, human data is somewhat limited. Diaz-Sanchez et al (1994, 1997 et al, 1997 et al) showed potentiation of immunoglobulin-E (Ig-E) when human nasal mucosa were exposed to diesel exhaust particles. When an

allergen is added with the diesel particles, the allergen specific Ig-E increases 50 fold. Diesel exhaust exposure has also been found to increase the number of mast cells, neutrophils and T-lymphocytes in bronchial tissue in humans (Diaz-Sanchez, 1994). Under high ambient concentrations short term exposure caused systemic and pulmonary inflammation in humans (Salvi, 1999). Fahy et al (2000) found that subjects, allergic to house dust mite, exposed simultaneously to house dust mite and diesel exhaust particles developed elevated chemokine levels via a mitogen activated protein kinase, which increases the onset of allergic respiratory onset. Casillas et al (1999) reviewed the evidence for the enhancement of allergic inflammatory responses and the possibility that macrophages produced upon exposure may cause generation of reactive oxygen species.

Finally, sodium arsenite is an environmentally ubiquitous chemical. With several (4) oxidation states it exists in numerous compounds and in a variety of forms. A naturally occurring element, it can be found in soil, water and ambient air. Sodium arsenite is toxic, and has been identified as a known human carcinogen (IARC, 1973). Arsenic has historically been used as a wood preservative and as an agricultural pesticide. This use and uncontrolled runoff from ore mining, have contaminated numerous sources of groundwater aquifers. The primary route of exposure for humans is ingestion of contaminated water and food stuffs (USEPA, EPA/600/R-98/042). Drinking water normally contains both the arsenate and arsenite forms of arsenic. This study used sodium arsenite which is a highly toxic, inorganic trivalent salt and is known to be present in drinking water. Sodium arsenite is used as a surrogate for arsenic in this study.

Arsenic is thought to affect immunologic responses. Several studies have looked at a variety of immunologic end points related to arsenic exposure. Arsenic treated T-

lymphocytes had an increase in cell death in mitogen stimulated cells (Goytia-Acevedo, 2003), a retardation of proliferation which was caused by delay of production and secretion of interleukin-2 (Galicia, 2003). A reduction of mitogenic stimulation was also seen with exposure (Gonsebatt, 1994). Mahata et al (2004) studied individuals with pre-existing arsenic induced skin lesions and found an increase in chromosomal aberrations after in vitro exposures of lymphocytes cultures of symptomatic subjects. This study concluded that asymptomatic sodium arsenite-exposed individuals have a lower sensitivity and susceptibility to induction of genetic damage compared to symptomatic individuals. In normal cell cycling progression from the S- to M-phase allows for proper cell division. Cell cycle progression from S- to M- phase was altered in arsenic-exposed individuals. The data showed a gender-related difference with mitogen stimulation and that chronic As ingestion decreased the rate of normal cell cycling, which should decrease cell proliferation but allows for chromosomal aberrations to increase (Gonsebatt, 1994). Vega et al (1999) utilizing electron microscopy, found altered cellular ultrastructure in Golgi apparatus, mitochodria, cytoskeleton and perinuclear membrane to be altered in arsenic treated peripheral blood mononuclear cells. Ostrosky-Wegman et al (1991) evaluated cell cycle kinetics between two groups of the same population, with differing levels of arsenic exposures, utilizing the in vitro lymphocyte proliferation assay. A group of chronically exposed individuals (n=13) compared to individuals with lower arsenic exposures (n=11) showed doubling of average time necessary for 100 mitoses of lymphocytes after incubation for 72 hours. Zhang et al (2003) treated two human cell lines with arsenite in a range of doses 0.1-40 μM. Proliferative capacity as measured by

LPA and cell viability analysis showed increased cellular growth at 0.1-0.5 μM and significant decreases when 0.5 μM was exceeded.

Our study sought to evaluate, in vitro, the immunotoxicity of B(a)P, diesel exhaust extract and sodium arsenite on treated T-lymphocytes as measured by LPA. We measured stimulated T-lymphocytes isolated from whole blood, treated with the three toxicants to establish dose response curves and comparative potencies as measured by the toxicant effects on the proliferative capacity of T-cell lymphocytes. Also we sought to determine whether an individual's intrinsic proliferative capacity was predictive of subsequent enhancement or suppressive effect following toxicant exposure. Finally we examined the feasibility of the current methodology and its potential application in field epidemiological studies.

Methods and Materials

Study Subjects

Thirty-eight subjects recruited in Chapel Hill, NC participated in this study. Ten subjects from a concurrent pediatric study (ages 8 - 18) and 28 adults participated. Adult study subjects were interviewed by phone, by the EPA onsite contractor (Westat Corp.), for potential participation in the study. The eligibility requirements for participants were as follows; age between 18 and 38 years, current non-smoker or ex-smoker (longer than five years) restricted from participating if blood donations exceeded 100 ml within the last 18 months, and absence of illness for four weeks prior to visit. The subjects agreed to abstain from alcohol, anti-inflammatory drugs and vitamins C and E for forty-eight hours prior to the clinic visit. The subjects were also asked to avoid smoky or polluted environments for twenty-four hours prior to the clinic visit.

Clinic visits consisted of administration of informed consent, a dietary/lifestyle questionnaire and a future storage of biological sample consent form followed by blood and urine collection. Potential study subjects were given a short verbal presentation and asked to read the prepared consent forms describing the study. Subjects were provided a copy of the consent form signed by them and by the principal investigator for their records. The subjects were asked to complete a short questionnaire, containing eighteen dietary/lifestyle questions. Upon completion of consents and questionnaire, a nurse drew four six- ml blood samples and a self administered urine sample were collected in a sterile container. All questionnaires and forms were reviewed and approved by the Institutional Review Board at University of North Carolina Medical School. All clinic staff and researchers were trained in human subject protection guidelines. This training is

required by the University of North Carolina for all persons engaged in research involving human subjects and pertains to the ethical conduct in research and protection of subjects.

Human Lymphocyte Isolation

Blood was collected in four acid-citrate dextrose (ACD) yellow top tubes (approximately 24 ml); (Becton Dickinson, NJ, Solution B). The anticoagulated tubes utilized a Vacutainer® Safety-Lok™ Blood Collection Set (21 G ¾ x 12" Becton Dickinson, NJ). Blood was transferred to 3-4 sterile 15 ml conical centrifuge tube and mixed with equal volume 1x Dulbecco's Phosphate Buffered Saline (D-PBS, Invitrogen NY). For each tube of blood used, 3 ml Isoprep® solution (2:1) Ficoll density medium (Robbins Scientific, CA) was pipetted into a separate 15 ml centrifuge tube. Diluted blood was carefully overlayed onto the Isoprep®solution, making sure not to break the Ficoll interface, centrifuged at 800xg (2100rpm) for 20-30 minutes according to the manufacturer's instruction. The peripheral blood mononuclear cells (PBMC) layer was carefully removed with a Pasteur pipette using a swirling motion, to remove cells that adhered to the centrifuge tube wall. This layer contained the white blood cells (white cloudy layer) in between plasma (top) and the red blood cell pellet (bottom). The PBMC were transferred to a 15 ml centrifuge tube and gently resuspended in 10 ml PBS. This was followed by centrifugation of cells at 250 x g (1200 rpm) for ten minutes. The supernatant was carefully removed and the lymphocyte pellet resuspended in 10 ml PBS. This washing procedure was again repeated. Lymphocytes were then resuspended in RPMI Medium 1640 without L-glutamine (Invitrogen, NY). Viability of cells was determined using the trypan blue (Gibco, NY) cell exclusion method. This method

involves placing a 1:1 mixture by volume of suspended cells to trypan blue. Using a pipette to mix thoroughly, the cells were placed on a hemacytometer with a cover slide. Viable cells will exclude the trypan blue dye and can be seen with a white nucleus surrounded by a blue membrane. Dead or damaged cell membranes will allow staining of cell nucleus with blue color. Cells were counted in all four quadrants utilizing a light microscope (Olympus, NY) and counts averaged. An aliquot was taken and based on the number of total viable cells, was diluted with RPMI to a final lymphocyte concentration of $1x10^6$ cells per ml of RPMI.

Preparation of Chemicals

Benzo[a]pyrene (B(a)P, Sigma, MO) was dissolved in reagent grade dimethylsulfoxide (DMSO, Sigma, MO) to a final concentration of 0.2 M. Diesel Exhaust Extract 1650 (DEE); (NIST, MD) dissolved in 1.2 ml of dichloromethane was solvent exchanged with DMSO to a final concentration of 0.2 M. The nominal concentration of DEE was based upon approximating the entire solid mass to representative polycyclic aromatic hydrocarbon B(a)P with a molecular weight of 252.3. These toxicants were aliquoted into amber vials and stored at –80°C. Sodium arsenite was prepared daily in deionized water, from a concentrated 0.5 M stock solution, stored at room temperature. Sodium arsenite was diluted with deionized, distilled water to final concentrations of 0.2, 2.0 and 20 µM. Lyophilized PHA was rehydrated by addition of 10 ml sterile distilled water to 2 mg/ml, and stored at -20° C. Previously prepared cell culture media was thawed from –80° freezer: RPMI 76% (Gibco, NY no L-glutamine), fetal bovine serum 20% (Gibco, NY, FBS, Heat inactivated), 2% Penicillin-Streptomycin (Gibco NY, 5000

u/ml: 5000 ug/ml), 2% L-Glutamine (Gibco, NY 200mM) by volume. 160 ml of RPMI without L-glutamine was added to 100 ml FBS to a final concentration of 20%, 10 ml Penicillin-Streptomycin and 10 ml L-glutamine, to final concentrations of 2.0%, were added prior to incubation. Cell culture media was filtered with 500 ml filter pack (Corning, NY).

Dosing and Plating of Cells

Each subject's lymphocytes acted as their own control (baseline) to evaluate subsequent proliferative capacity following the addition of a mitogenic stimulant phytohemagglutinin (PHA Gibco, NY). The controls were as follows; 1) media control-cells plus media (RPMI), 2)PHA/ media control- cells, media and PHA, 3) DMSO control-cells, DMSO and media, 4) PHA/DMSO control-cells, PHA, DMSO, and media. To facilitate homogeneous mixing of cells, toxicants and media were placed in Eppendorf tubes and mixed with gentle vortexing, to ensure adequate mixing prior to plating . 350µl (0.35 ml) of the $1x10^6$/ml cells were placed in thirteen 0.5 ml Eppendorf tubes. Also doses of three toxicants B(a)P, DEE at 0.2, 2.0 and 20 µM and sodium arsenite 0.02, 0.2 and 2.0 µM were added. To stimulate cellular proliferation PHA (22 µl; 2 mg/ml) was added to all tubes except control tubes 1 & 3. To facilitate plating of the three toxicants at the three different dose levels, nine additional tubes with 0.35 ml of cells, PHA and cell culture media added such that the total volume in each well was 500 µl irrespective of treatment. Immediately prior to plating each tube toxicant was added. Tubes were gently vortexed to mix thoroughly and 100 µl was pipetted into triplicate wells of a 96 well plate (Costar, MA).

Measurement of Lymphocyte Proliferation

Lymphocytes were incubated for 68 h at 37°C in a 5% CO_2 atmosphere (Forma Scientific Steri-Cult 200). Changes in any cellular proliferation was quantitated following addition of 40 µl CellTiter 96® Aqueous One (Promega, CA) MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] which is reduced to formazan by cells that are metabolically active (Barltrop, 1991). After addition of the AQ One, incubation for 4 h, quantification was determined by reading the absorbance on microplate reader (Anthos, Salzburg, Austria) at 490 nm.

Cytotoxicity Study

To evaluate the toxicity of B(a)P, DEE and sodium arsenite for purposes of establishing doses that were not cytotoxic each well containing lymphocytes was treated at each dose in the absence of PHA for 68 hrs and compared to cells with no toxicant treatment.

Data Inclusion Criteria

A criterion for inclusion of data for subsequent analysis was inter-well variability < 20% for each dose of each treatment (triplicate wells), and < 20% variability between the two controls, incorporating the two solvents used to dilute the toxicants. The control for B(a)P and DEE contained DMSO; control for sodium arsenite contained water. Each control contained the solvents; cells, RPMI, duplicated with and without PHA to assure mitogenic stimulation had occurred. Sixteen out of 38 subjects met these stringent criteria

for all toxicants at all doses. Data in the figures reflect either the n = 16, or all data n = 38, i.e. regardless of whether we had data for all doses and toxicants.

The baseline data was rank ordered and then equally divided into two groups (group 1 those with higher baseline proliferative capacity; group 2 those with lower baseline proliferative capacity). This sorting of data enabled us to assess whether there were different responses by group.

Statistical Analysis

Data analyses were conducted for lymphocyte proliferative responses for each subject. Comparisons between subjects' proliferative changes, due to toxicant treatment, were evaluated by grouping according to age, gender and mean response. Mean proliferative response for n=38 served as division point for data into group 1 (above mean) and group 2 (below mean) and for n= 16 group A (above mean) and group B (below mean). Coefficients of variation (CV) were calculated, to compare variations of the distributions, as standard deviation divided by mean. Student-t tests were used to compare the actual difference between two means in relation to the variation in the data (expressed as the standard deviation of the difference between the means). Statistical significances were established with alpha set at 0.05, 0.01 and 0.001. Data were assumed to be normally distributed.

Statistical analysis was done using Excel® software from Microsoft Office Suite 2003 (Redmond WA). Included are averages, correlation coefficients, standard deviations, student t tests, (paired, homoscedastic, and heteroscedastic). Graphical representations (figures 4, 5, 6) Excel ® other representations are Prism software version 3.02 (2000)

from GraphPad Software, Inc. (San Diego, CA). The effects of toxicant treatments on human T-lymphocytes were measured by absorbance on an optical plate reader. Viable T-lymphocytes metabolize CellTiter 96® Aqueous One into a light absorbing formazan dye, and can be quantitatively measured by alteration in absorbance. Toxicant treated mitogen stimulated lymphocytes are compared to untreated mitogen stimulated lymphocytes acting as controls.

3. Results

Comparative Cytotoxicity

Figure 1 represents the results of cytotoxicity testing for all three toxicants at three dose levels. Responses are shown as optical densities with mean +/- standard deviations and p-values. To determine cytotoxicity, cells with toxicant treatments but no mitogenic stimulation (PHA) were evaluated. Unstimulated cells are used when testing for cytotoxicity because normal cell function must be present. Any alteration such as stimulated clonal proliferation might mask the true cytotoxicity of the compound. Suppression of responses due to treatments were compared to controls for significant differences. Only the highest dose of As 20 μM $p \leq 0.001$, (0.304 ± 0.006; mean ± S.D.) was toxic. Based on this observation, we reduced the highest dose of sodium arsenite from 20 to 2.0 μM. Thus the following doses were chosen for subsequent immunotoxicity testing: 0.2, 2.0 and 20 μM for B(a)P and DEE and 0.02 and 0.2 and 2.0 μM for sodium arsenite.

Comparative Potency

Figure 2 shows the data from 16 subjects for which we had data for each toxicant and dose. Effect of treatment was measured by changes in proliferation response,

normalized to baseline levels and quantitated as a percentage of the control or baseline level. B(a)P ($p \leq 0.001$) and DEE ($p \leq 0.001$) were significantly different from sodium arsenite at the 2.0 μM dose, but not from each other, B(a)P (99.2 ± 33.0) and DEE (106.7 ± 22.4). This illustrates that sodium arsenite is more toxic 1.73 times more toxic than B(a)P and 1.84 times more toxic than DEE. No significant differences from control levels were observed at the lower two doses irrespective of treatment.

We observed a greater inhibition of cellular proliferation for sodium arsenite treated lymphocytes than for B(a)P or DEE. The sodium arsenite suppressed cellular proliferation at a dose ten times lower (2.0 μM) than B(a)P and DEE (20 μM). Mean lymphocyte proliferative capacities compared to controls of 100% for B(a)P and DEE at 2.0 μM were 99.2% and 106.7% respectively compared to 57.9 % suppression of proliferation for sodium arsenite at the 2.0 μM level.

Dose Dependent Effects on Lymphocyte Proliferation

Figure 3 represents linear regression and Figure 4 mean data from all subjects (n=38), regardless of whether each individual had data for all three doses and toxicants. B(a)P and DEE at the 20 μM doses were significantly different from the control levels ($p \leq 0.01$). Sodium arsenite, at the 2.0 μM level, was different statistically significant from the control level ($p \leq 0.001$). When compared to control levels a slight increase in proliferative capacity was seen in the 2.0 μM and the 0.2 μM DEE (102.6% and 103.1%). The coefficient of variation for individuals at the control levels for all the subjects (n=38) was 27.7%. This variation between individuals was lower following all three treatments

except at the highest dose where more variability in responsiveness was observed i.e. B(a)P, 33.3%; DEE, 37.1%; Sodium arsenite, 43% respectively.

Interindividual responses to treatment

Mean values of proliferative response changes (Figure 2 and 3) following treatment do not provide useful information on individual responses to treatment. Figures 5, 6, and 7 represent individual responses to B(a)P, DEE and sodium arsenite. They also illustrate the equally diverse range of stimulative and suppressive effects of BaP, DEE and sodium arsenite at the two lower doses. About an equal number of individuals showed stimulated proliferation as showed suppressed proliferation. At the two lowest doses for all three chemicals the immunotoxicant effects were almost equally divided between those individuals responding with enhanced stimulatory effects and those responding with an immunosuppressive effect (figures 5, 6, 7). For example, the two lowest doses of B(a)P, 0.2 μM, and 2.0 μM, showed 44% and 56% of the individuals had immunosuppressive response respectively; DEE, 0.2 μM, and 2.0 μM, 50 and 50 percent respectively; sodium arsenite, 0.02 μM, and 0.2 μM 60% and 40% of the individuals' lymphocytes had a immunosuppressive response respectively. In contrast at the highest dose level 81%, 62%, 94% of the individuals demonstrated an suppressive effect on proliferation at the highest doses of BaP, DEE and sodium arsenite respectively. In contrast the lymphocytes from the majority of individuals show a clear immunosuppressive response at the 2.0 μM dose of sodium arsenite.

Differences in intrinsic proliferative capacity by age and gender

Figure 8 illustrates the cellular proliferative capacity of the subjects in the presence of PHA. The figure presents the data with the subjects from each data set (n=38 all data meeting the data analysis criteria) and (n=16 which included data from only those individuals that had data for all three toxicants at all three doses). After the intrinsic data was rank ordered with respect to an individual's cell proliferation in the presence of PHA, we divided the data into two equal groups; those with high proliferative capacity (group 1, n = 18; group A, n = 8) and those with low capacity to proliferate (group 2, n = 18 ; group B, n = 8). Differences between baseline proliferative capacity were statistically different from each other [group 1 & 2; n=38, $p \leq 0.001$; (group A & group B; n=16, $p \leq 0.001$)]. Figure 9 shows that pediatric subjects, (children less than 18 years old) were not statistically different from the adults at the $p \leq 0.05$ level. Proliferative responses of men were statistically different (higher) from women ($p \leq 0.05$, 107.6 ± 30.39).

Figure 8 illustrates clear differences between the two groups of individuals' responses at the highest dose for all three toxicants. Group 1 was significantly lower ($p \leq 0.05$, $p \leq 0.05$ $p \leq 0.001$) than group 2 at 20 μM B(a)P and DEE and As at the 2.0 μM dose respectively.

Similarly, Figure 10 demonstrates that group A individuals (those with a high intrinsic proliferative capacity relative to group B) respond to treatment by decrease in proliferative capacity following treatment with all three toxicants at all dose levels. In contrast the group with a lower proliferative capacity (group B) shows stimulatory

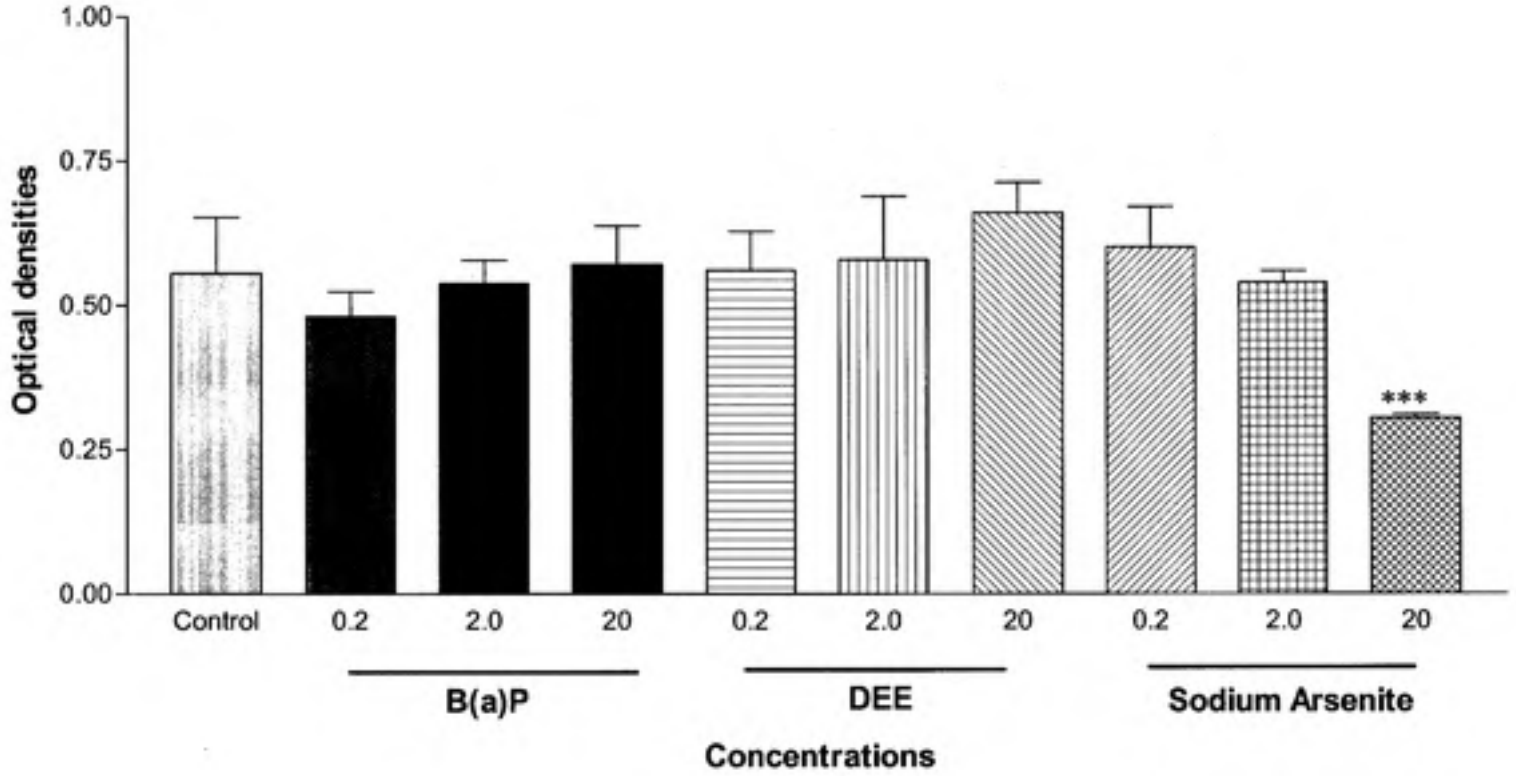


Figure 1. Cytotoxicity testing of human T-lymphocytes treated *in vitro* with B(a)P, DEE and sodium arsenite. Treated cells were exposed in cell culture for 68 hrs to B(a)P (0.2, 2.0, 20 μM), DEE (0.2, 2.0, 20 μM), or sodium arsenite (0.02, 0.2, 2.0 μM). Cellular proliferation was evaluated by incubation of cell cultures with Aqueous One ® for 4 hrs and measuring optical absorbance ($\lambda = 492$ nm). Decrease in the response of T-lymphocytes to PHA stimulation were determined to be statistically significant by Student's *t*-test: *** $p \leq 0.001$.

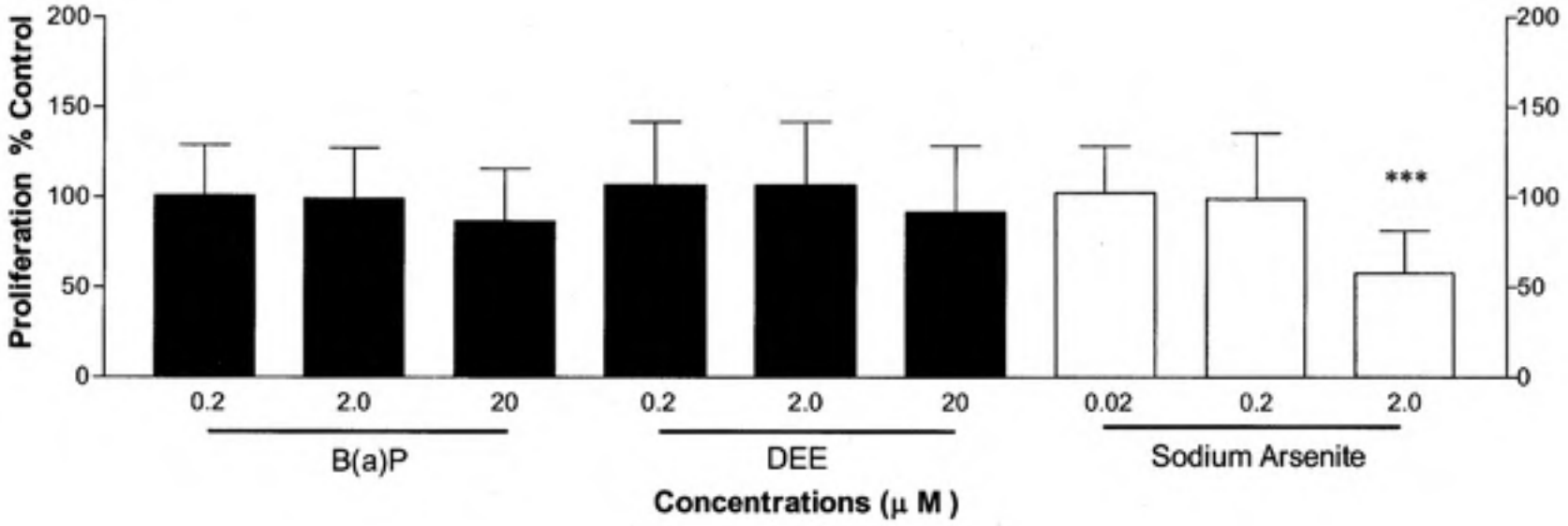


Figure 2. Comparative potency. *In vitro* proliferative responses (n = 16) of human T-lymphocytes (mean ± SD) presented as percent of control cells. Control and treated cells were stimulated with PHA to induce a cellular proliferation. Treated cells were exposed in cell culture for 68 hrs to B(a)P (0.2, 2.0, 20 μM), DEE (0.2, 2.0, 20 μM), or sodium arsenite (0.02, 0.2, 2.0 μM). Cellular proliferation was evaluated by incubation of cell cultures with Aqueous One ® for 4 hrs and measuring optical absorbance (λ = 492 nm). Decreases in the proliferative response of T-lymphocytes were determined to be statistically significant by Student's *t* test: *** $p \leq 0.001$.

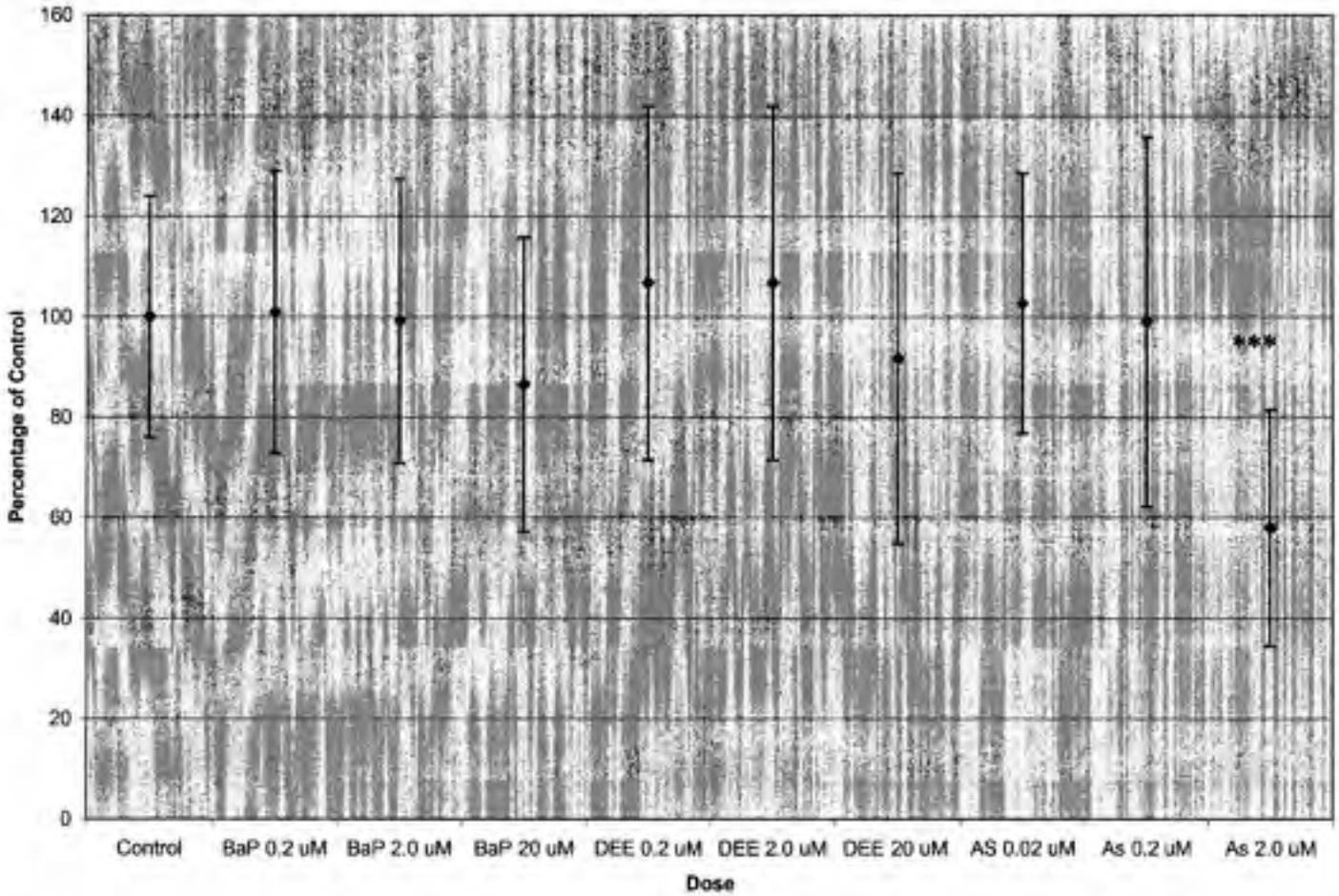


Figure 3. Comparative potency of B(a)P, DEE and sodium arsenite. *In vitro* proliferative responses (n = 16) of human T-lymphocytes (mean ± SD) presented as percent of control cells. Control and treated cells were stimulated with PHA to induce a cellular proliferation. Treated cells were exposed in cell culture for 68 hrs to B(a)P (0.2, 2.0, 20 μM), DEE (0.2, 2.0, 20 μM), or sodium arsenite (0.02, 0.2, 2.0 μM). Cellular proliferation was evaluated by incubation of cell cultures with Aqueous One ® for 4 hrs and measuring optical absorbance (λ = 492 nm). Decreases in the proliferative response of T-lymphocytes were determined to be statistically significant by Student's *t*-test: *** $p \leq 0.001$.

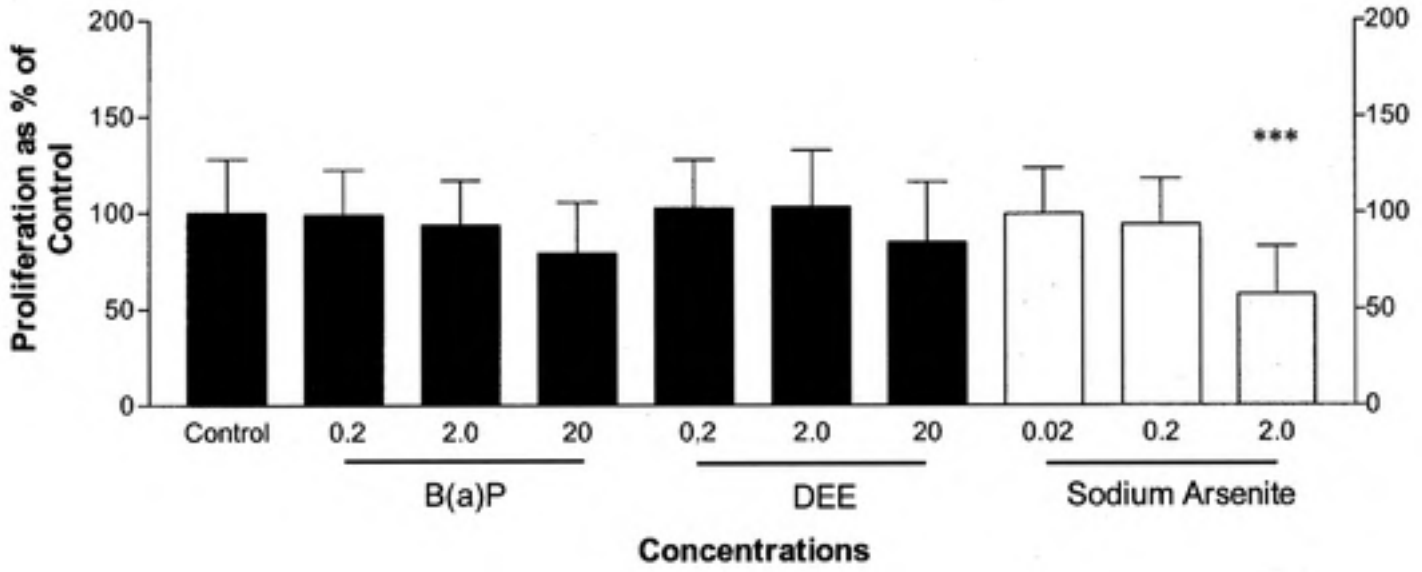


Figure 4. Dose dependent proliferative capacity. *In vitro* proliferative responses (n = 38) of human T-lymphocytes (mean ± SD) presented as percent of control cells. Control and treated cells were stimulated with PHA to induce a cellular proliferation. Treated cells were exposed in cell culture for 68 hrs to B(a)P (0.2, 2.0, 20 μM), DEE (0.2, 2.0, 20 μM), or sodium arsenite (0.02, 0.2, 2.0 μM). Cellular proliferation was evaluated by incubation of cell cultures with Aqueous One ® for 4 hrs and measuring optical absorbance (λ = 492 nm). Decreases in the proliferative response of T-lymphocytes were determined to be statistically significant by Student's *t* test: *** $p \leq 0.001$.

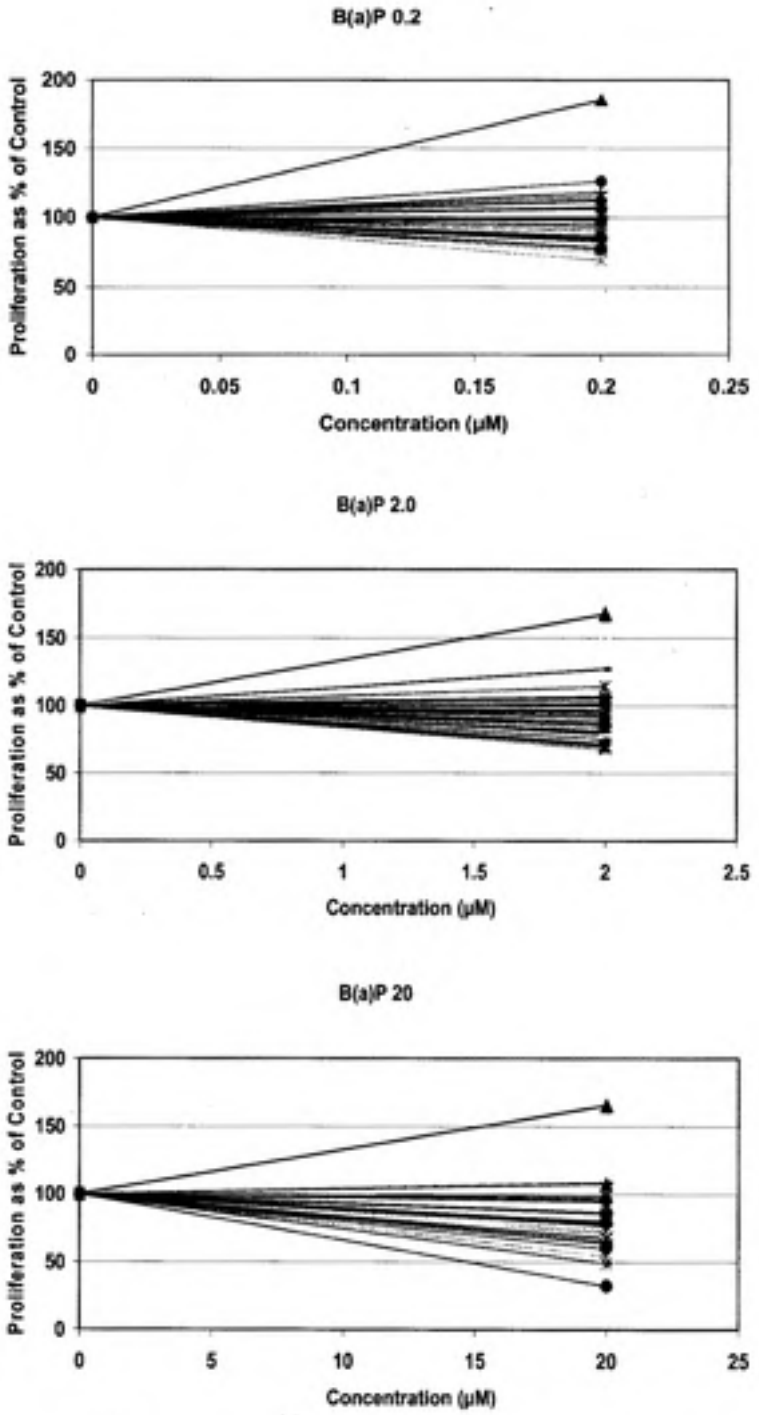


Figure 5. Inter-individual differences in proliferative response to B(a)P. *In vitro* proliferative responses (n = 16) of human T-lymphocytes results are presented as percent of control cells. Treated cells were exposed in cell culture for 68 hrs to B(a)P (0.2, 2.0, 20 μM). Cells were stimulated with PHA to induce a cellular proliferation. Cellular proliferation was evaluated by incubation of cell cultures with Aqueous One ® for 4 hrs and measuring optical absorbance (λ = 492 nm).

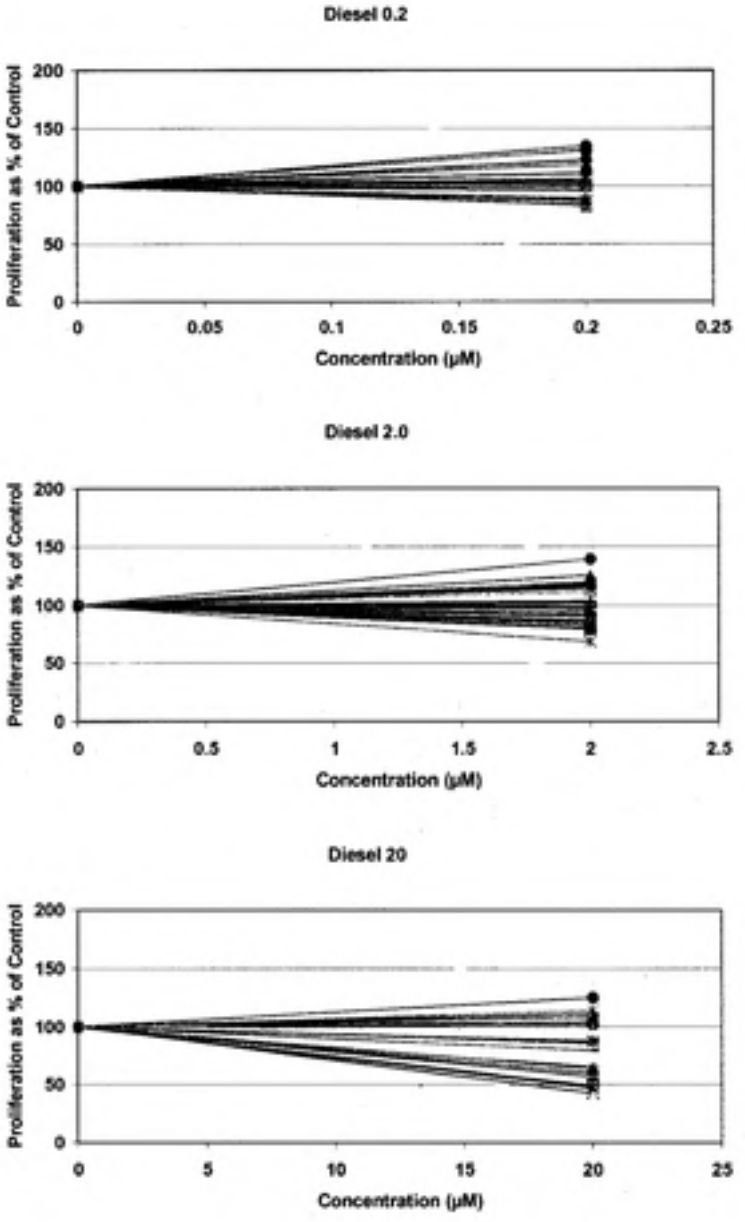


Figure 6. Inter-individual differences in proliferative response to DEE. *In vitro* proliferative responses (n = 16) of human T-lymphocytes results are presented as percent of control cells. Treated cells were exposed in cell culture for 68 hrs to DEE (0.2, 2.0, 20 µM). Cells were stimulated with PHA to induce a cellular proliferation. Cellular proliferation was evaluated by incubation of cell cultures with Aqueous One ® for 4 hrs and measuring optical absorbance (λ = 492 nm).

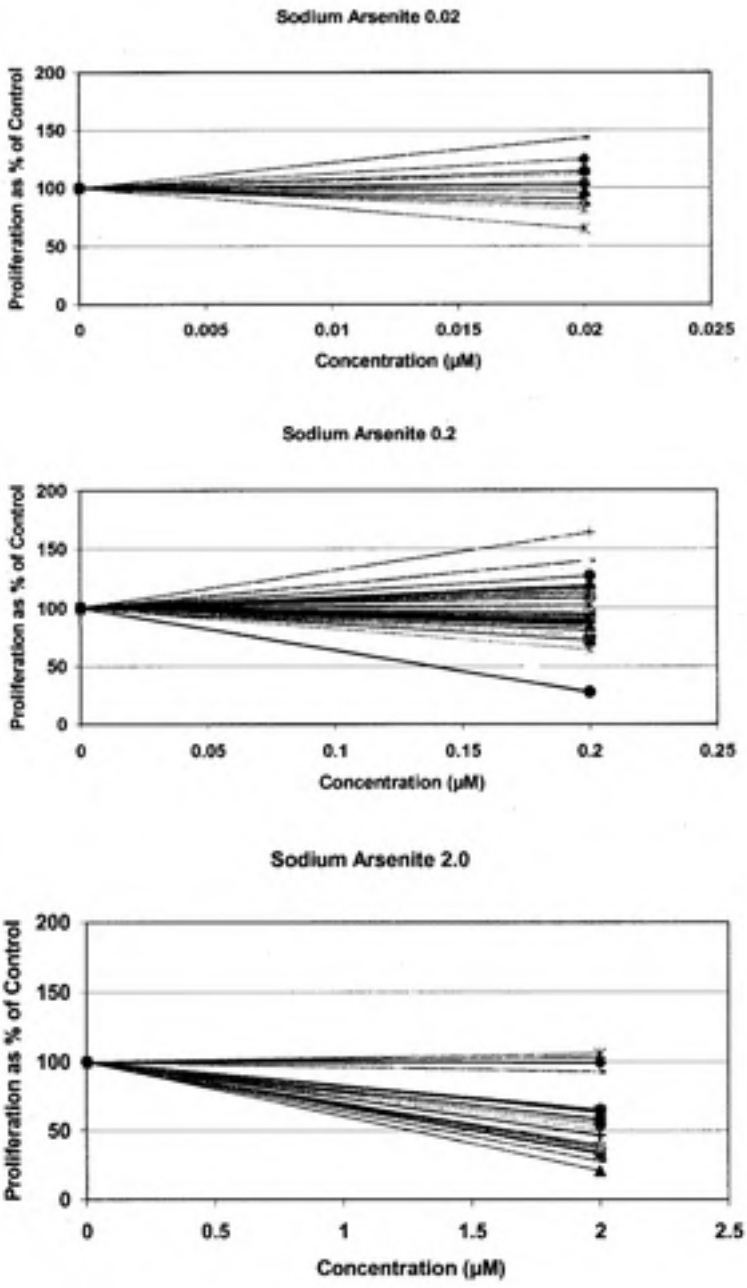


Figure 7. Inter-individual differences in proliferative response to sodium arsenite. *In vitro* proliferative responses (n = 16) of human T-lymphocytes results are presented as percent of control cells. Treated cells were exposed in cell culture for 68 hrs to sodium arsenite (0.02, 0.2, 2.0 µM). Cells were stimulated with PHA to induce a cellular proliferation. Cellular proliferation was evaluated by incubation of cell cultures with Aqueous One ® for 4 hrs and measuring optical absorbance (λ = 492 nm).

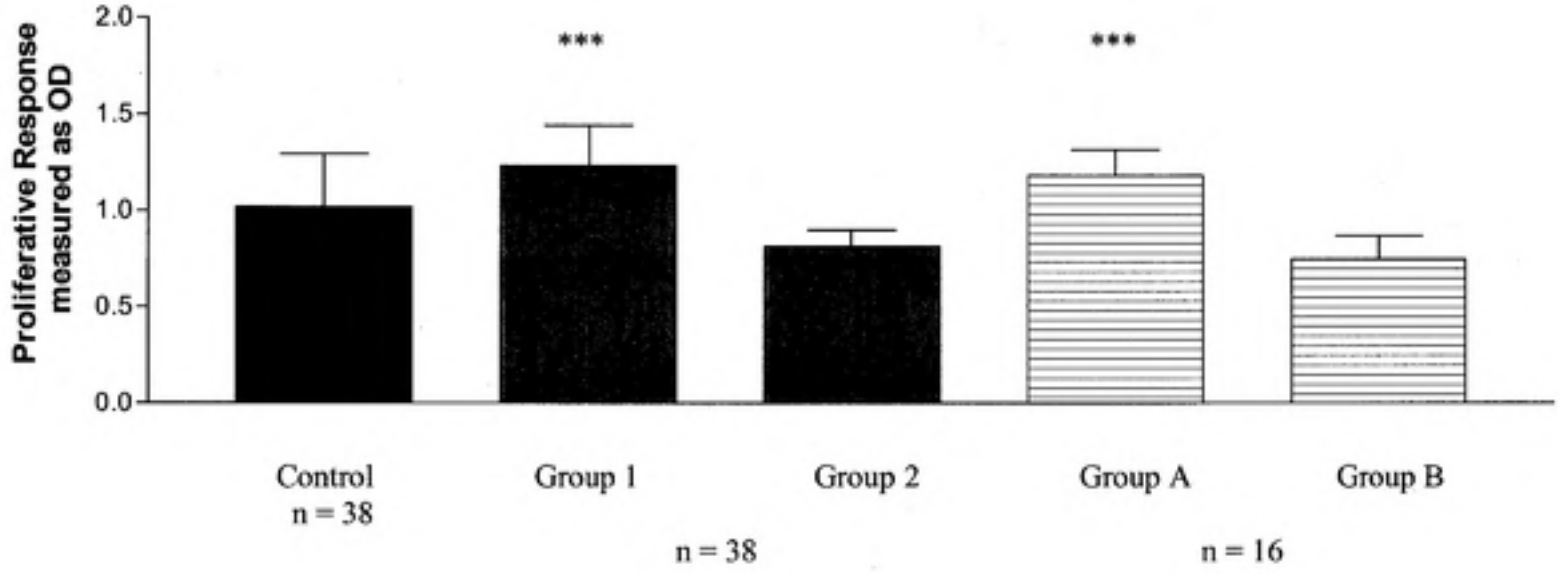


Figure 8. Intrinsic proliferative capacity. *In vitro* proliferative responses of human T-lymphocytes (mean ± SD) presented as optical densities. Cells incubated without treatment for 68 hrs; cells were stimulated with PHA to induce a cellular proliferation. Cellular proliferation was evaluated by incubation of cell cultures with Aqueous One ® for 4 hrs and measuring optical absorbance ($\lambda = 492$ nm). Decreases in the proliferative response of T-lymphocytes were determined to be statistically significant by Student's *t*-test: *** $p \leq 0.001$.

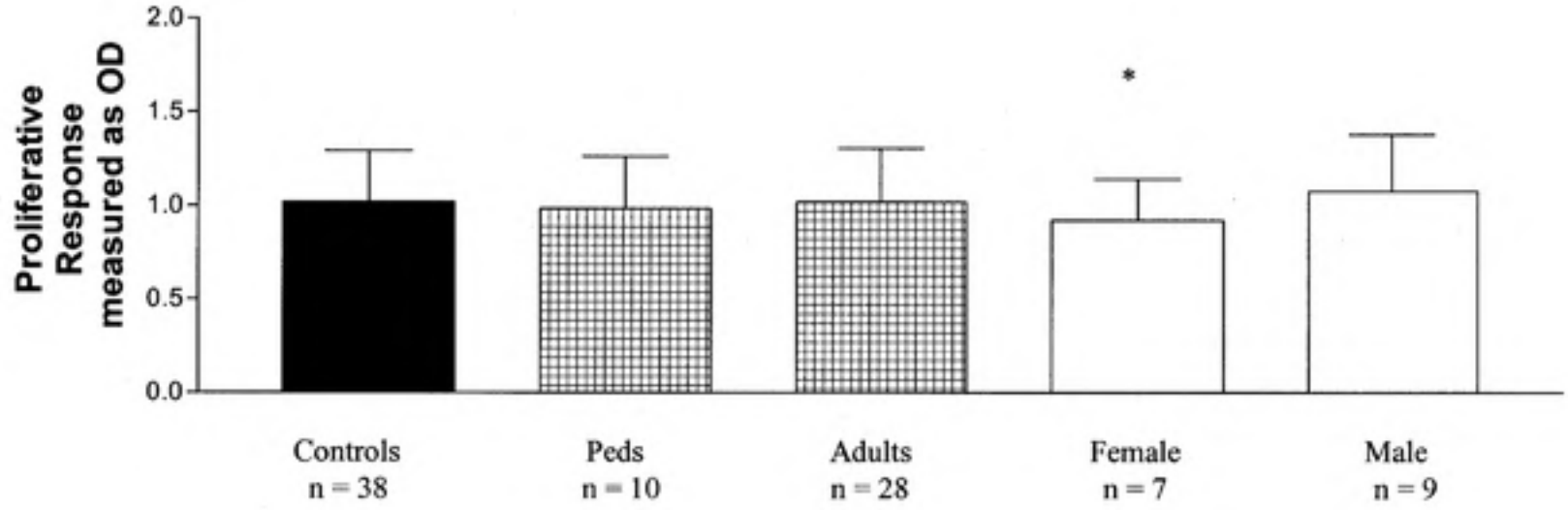


Figure 9. Intrinsic proliferative capacity in children compared to adults; and females intrinsic proliferative capacity compared to males. *In vitro* proliferative responses of human T-lymphocytes (mean ± SD) presented as optical densities. Cells incubated without treatment for 68 hrs; cells were stimulated with PHA to induce a cellular proliferation. Cellular proliferation was evaluated by incubation of cell cultures with Aqueous One ® for 4 hrs and measuring optical absorbance ($\lambda = 492$ nm). Decreases from controls in the proliferative response of T-lymphocytes were determined to be statistically significant by Student's *t*-test: * $p \leq 0.05$.

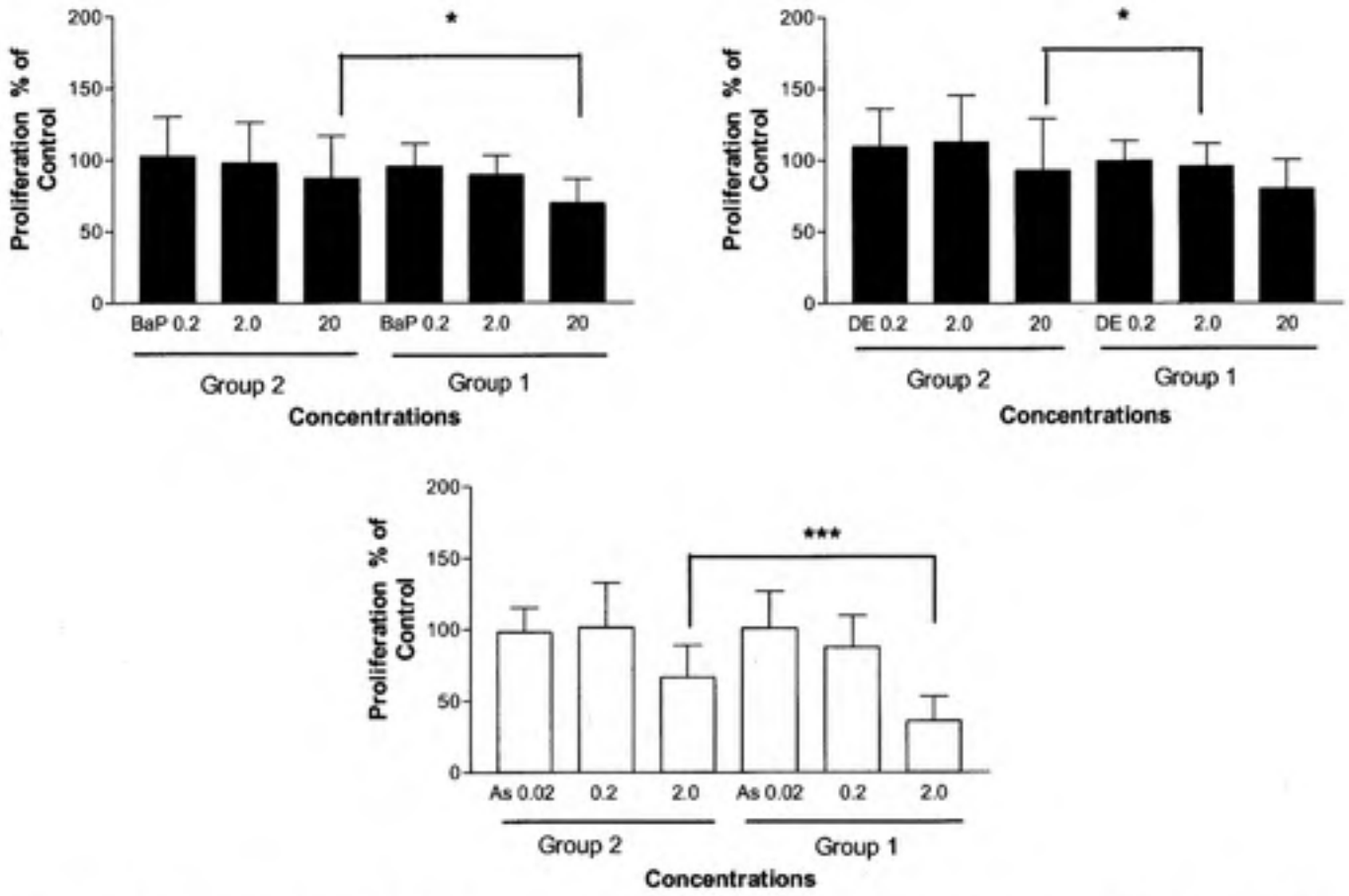


Figure 10. Comparisons in responses between two groups separated by mean (n=16). Group1 (n = 8), above mean represents high intrinsic proliferative capacity; group 2 (n= 8) below mean represents low intrinsic proliferative capacity. *In vitro* proliferative responses of human T-lymphocytes (mean ± SD) presented as percentage of controls. Treated cells were exposed in cell culture for 68 hrs to B(a)P (0.2, 2.0, 20 µM), DEE (0.2, 2.0, 20 µM), or As (0.02, 0.2, 2.0 µM). Cells were stimulated with PHA to induce a cellular proliferation. Cellular proliferation was evaluated by incubation of cell cultures with Aqueous One ® for 4 hrs and measuring optical absorbance ($\lambda = 492$). Decreases in the proliferative response of T-lymphocytes were determined to be statistically significant by Student's *t* test: * $p \leq 0.01$; *** $p \leq 0.001$.

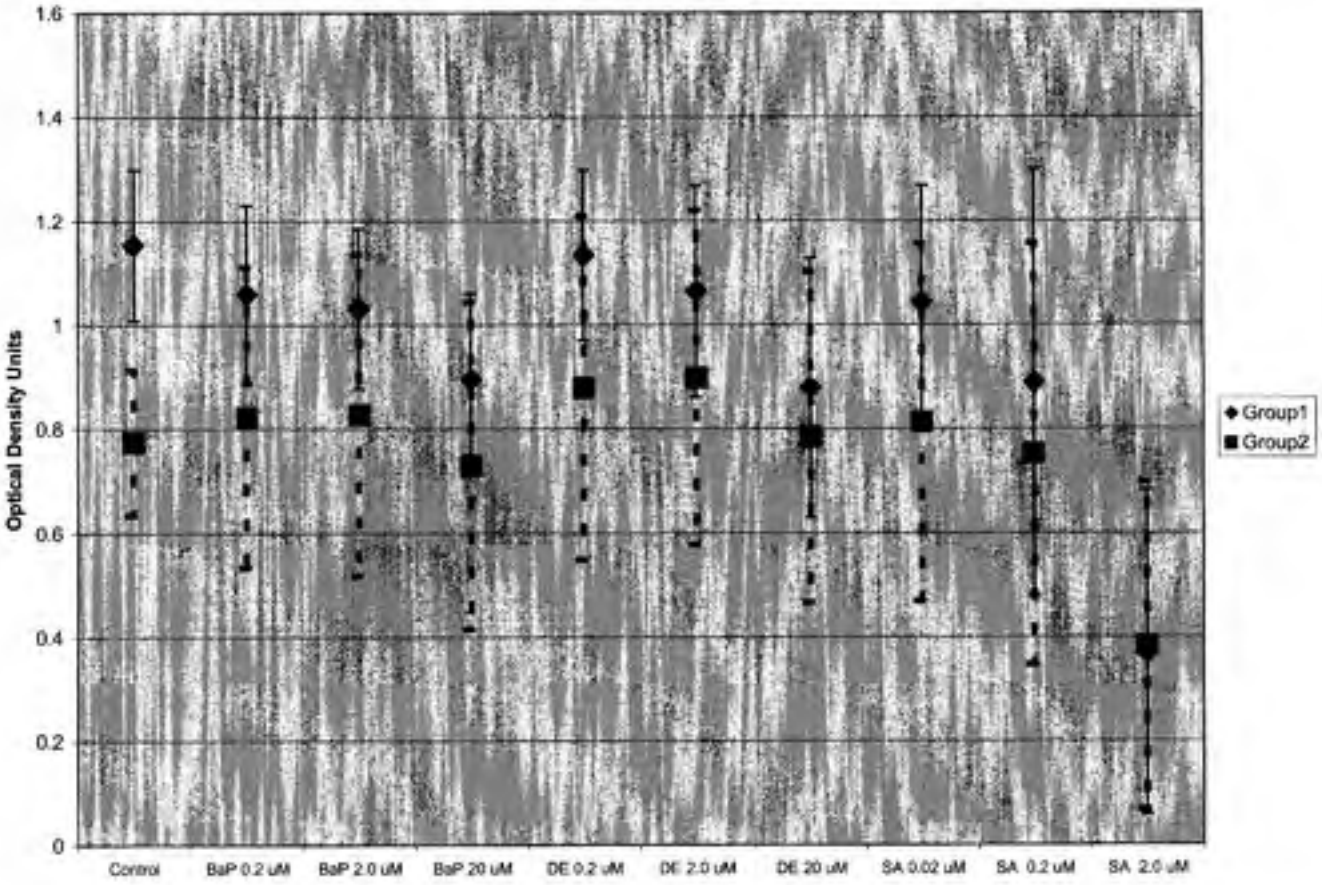


Figure 11. Differential effects of toxicants stratified by intrinsic proliferative capacity. *In vitro* proliferative responses (n = 16) of human T-lymphocytes presented as optical densities. Treated cells were exposed in cell culture for 68 hrs to B(a)P (0.2, 2.0, 20 μM), DEE (0.2, 2.0, 20 μM), or sodium arsenite (SA) (0.02, 0.2, 2.0 μM). Cells were stimulated with PHA to induce a cellular proliferation. Cellular proliferation was evaluated by incubation of cell cultures with Aqueous One ® for 4 hrs and measuring optical absorbance (λ = 492 nm).

4. Discussion

Our work examined the in vitro immunotoxicity of three environmentally relevant compounds, B(a)P, DEE and sodium arsenite as assessed by evaluating effects on PHA stimulated cellular proliferation of T-lymphocytes isolated from whole blood of 38 individuals (10 children, 28 adults). The goals of our study were to evaluate the cytotoxicity, relative potency and dose-dependent effects of these three chemicals. We measured the effects of treatment with toxicants by changes in proliferative capacity relative to controls. We also investigated whether a person's baseline proliferative capacity predicted the extent of immunotoxicity from treatment with the toxicants and whether this sensitivity was dose or chemical dependent. By rank ordering the data above and below the mean, we compared responses to mitogenic stimulation between high (higher than mean) proliferative capacity individuals to individuals with low (lower than mean) proliferative capacities. Finally, the applicability of the current assay and its usefulness in field epidemiological studies is addressed.

Our study examined in vitro effects of sodium arsenite, as a surrogate for arsenic, on the proliferative capacity of T-lymphocytes at a dose range of 0.02 μM to 2.0 μM. The levels that we used for sodium arsenite approximated actual blood concentrations of arsenic exposed individuals. Wu et al (2001) looked at of individuals chronically exposed by drinking water and reported arsenic blood levels ranging from 0.058 μM (equivalent 4.32 μg/L) to 0.62 μM (equivalent 46.5 μg/L). Pi et al (2000) found blood arsenic levels as high as 0.562 μM (equivalent 42.1 ng/ml) in drinking water exposed individuals in China. The current study found statistically significant decreases in proliferative capacity with sodium arsenite at the 0.02-2.0 μM dose levels. This may be a

good biomarker of immune effects in individuals exposed to arsenic through their drinking water, if blood levels approached levels as detected in the Pi et al and Wu et al studies. Because B(a)P and DEE are metabolized to toxic intermediates first, direct comparisons of the doses we used in this study versus internal measures are not possible. B(a)P, DEE are air and water contaminants and are thought to exert their toxicity following metabolic activation by different mechanisms from arsenic. Because the immune system is a common target for toxic effects of a number of poly aromatic hydrocarbons (PAH), most notably B(a)P, we were interested in ascertaining T-lymphocyte mediated cell response based on an application frequently utilized by researchers, industry and the USEPA, in Tier 1 immunotoxicity screens.

Cytotoxicity, Comparative Potency and Dose Dependent Effects

First, a study to evaluate the cytotoxicity of the three toxicants B(a)P, DEE and sodium arsenite at three doses was conducted , only the 20 μM sodium arsenite was cytotoxic. Assessment of the relative potency of the toxicants showed that when compared on an concentration basis, sodium arsenite treatment (2.0 μM) produced in a statistically significant, immunosuppressive effect of control compared to B(a)P and DEE treatments. Dose-dependent decrease in proliferative capacity relative to the control was observed for all three chemical treatments; however these responses were statistically significant at the highest dose only. These doses are similar to doses used in other lymphocyte proliferation assays (Davila, 1996; Gonsebatt, 1994; Meltz 1981). Davila et al (1996) looked at B(a)P dose ranges from 0.01 μM to 10 μM, and their effect on T-lymphocytes and saw the greatest effect at the high dose (10 μM). Our study saw a

statistically significant decrease in proliferation only at the 20 μM concentration of B(a)P and DEE.

In Davila et al individual immunosuppressive effects ranged from 17%- 65% compared to baseline. The mean response of all individuals resulted in 20.8% suppressive effect of proliferation for the 20 μM dose. The current study, with a much larger number of subjects, resulted in a smaller range of proliferation of control and is possibly a better representation of the general population would respond with treatment. In the Davila study one or two individuals with high or low proliferative responses could have skewed the data to give the larger range of proliferative response. Another reason for the decreased percentage proliferation could be in the methodologies used i.e. radioactive nucleotide incorporation ([^{3}H]-thymidine) vs. absorbance, although the [^{3}H]-thymidine was in good agreement with absorbance when tested within the same experiment (Gieni 1995).

Gonsebatt et al (1994) using mitotic indices as a measure of proliferation of T-lymphocytes and observed the greatest dose-related inhibition of proliferation at the highest dose of arsenic. Treatment of lymphocytes with trivalent arsenite at concentrations from 0.001 to 0.1μM, inhibited mitosis from 101 % at the low dose to 56% of control values at the high doses. Meltz (1981) looked at baboon lymphocytes and the effect of diesel exhaust extract on proliferative effects (by measuring thymidine incorporation i.e. proliferation) at 0.8, 4.0 and 20 μM and saw the greatest effect at 20 μM dose. At the 0.8 and 4.0 μM dose uptake of thymidine was greater than controls. The suppression of cellular proliferation induced by diesel extracts by the 20 μM dose ranged in the Meltz et al study from 1-20% of the control values. This is similar to the

suppressive effects we observed at the high dose (8.31- 15.3 from both sets of data n=38, n=16 respectively).

Individual Intrinsic Proliferative Capacity

Beyond a look at the relative comparative potency as measured by immunotoxic responses, of interest was a closer inspection on an individual rather than group response. Our study then examined whether the sensitivity and or responsiveness to subsequent treatment were correlated with an individual's baseline proliferative capacity prior to treatment. Clearly, at all doses but most notably at the lower doses, there appeared to be striking inter-individual differences in response to toxicant treatment (Figures 5, 6, 7). Interestingly, at the two lowest doses for all three chemicals the immunotoxicant effects were almost equally divided between those individuals responding with enhanced stimulatory effects versus those responding with an immunosuppressive effect (Figures 5, 6, 7). These individual differences are not apparent when data is combined into group means (Figure 4). For example, for the n = 16 group following DEE (20 μM) treatment 10/16 of the individuals and B(a)P (20 μM), 13/16 of individuals responded to treatment with decrease in proliferative capacity in stark contrast to 15/16 upon treatment with (2.0 μM) sodium arsenite. Clearly, the ten fold lower dose of sodium arsenite and the number of individuals with suppression of proliferation are both of importance.

Inter-individual Variability

To ascertain the extent to which intrinsic (only mitogenic stimulation, no treatment) proliferative capacity affected the magnitude of response differences, we rank ordered the 38 individuals and divided them into a two equal groups; one with higher intrinsic proliferative capacity group 1 versus group 2 with a lower proliferative capacity and group A versus group B (groups described in results). This rank ordering was based on averages of the two controls discussed previously. Statistically significant differences in intrinsic proliferative capacity for each of these two groups were observed. This suggests a differential effect on toxicant treatment which depends on the individuals' lymphocytes inherent ability to proliferate in the absence of a treatment. There were no differences between the proliferative capacities in the children vs. adults but there was a statistically significant difference between baseline proliferative capacity in males and females.

The variability in the group whose intrinsic proliferation was higher (group1) when compared to the group with lower intrinsic proliferative capacity (group 2) was significantly reduced. This implies that the two groups were responding differently to the treatment. This is in agreement with the Vega et al (2004) study which demonstrated that activated lymphocytes were more susceptible to effects of treatment with sodium arsenite than resting lymphocytes. The study found no dose to be statistically different in males, illustrating no effect from sodium arsenite treatments in male subjects, contrasted with female subjects where significant differences were observed between the 0.1 and 1.0 μM

doses. This shows the greater susceptibility of lymphocytes derived from females to treatment with sodium arsenite. Proliferation was measured by thymidine uptake with untreated lymphocytes as controls.

In our study, grouping of data based on initial intrinsic effects (previously discussed) for those individuals treated with B(a)P (20 μM) and sodium arsenite (2.0 μM) with a high baseline proliferative capacity were significantly different compared to those with low proliferative capacity . Similarly at the 2.0 μM concentrations for DEE those individuals with a high baseline proliferative capacity compared to low responses were shown to be significantly different. This point is further emphasized (Figure 10) where individuals with high proliferative capacity (group 1) show a dose dependent immunosuppressive effect compared to with low baseline proliferative capacity (group 2). Figure 10 illustrates that for B(a)P, DEE and sodium arsenite there was a slight stimulatory effect for group 2 individuals at the lowest dose. The induction effect of T-lymphocyte proliferation at the lowest doses for all three toxicants is consistent with studies by Germolec et al (1996) who showed that treatment with low levels of sodium arsenite (0.001-0.01 μM) produced a slight increase in proliferation in keratinocytes. Evaluating the responses of the large group (n=38), the two lower B(a)P doses showed little immunotoxicity.

Many factors, as discussed in the introduction, can modulate an individual's response to environmental exposures. These include individual health and nutritional status, which can affect cell mediated immune status. On a molecular level, an individual's ability to repair damage that may result from exposures is a key factor when culturing conditions are longer than a few days. In vitro experiments require an

assessment of the experimental variability such that differences in responsiveness are minimally influenced by experimental conditions. Inconsistency in preparation of reagents and operator technique, particularly aliquoting accurately, can contribute additional variation. Individual health and nutritional status can add to variation by altering cell mediated immunity. Another cause of variability could be the different suppression mechanisms affected by the toxicants, which could be a key factor in the current study.

Variability is a key component in our study because we wanted to look at inter-individual variability within our study population. Several studies have looked at the variability issue. To evaluate the variability of a common assay at different labs, Froebel et al (1999) showed variability from 8 different labs conducting the lymphocyte proliferation assay utilizing PHA to range from 5-20% with an average of 15.5%. Methodological differences such as differing concentrations of stimulants, i.e. amounts of PHA utilized, and operational differences, i.e. time of blood draw and time elapsed before treatment, can all affect results. The average experimental variability within data (n=38) included in our study was 7.42%, derived from triplicate wells of controls and treatments. The use of a standard time protocol (sample collection to sample analysis) and experimental consistency adds to the marked improvement in data variability. Molls et al (1999) reports an analytical variation of 4-5% when they looked at thirty women's proliferative capacity with PHA. The study looked at fifteen young and fifteen old women to look at effects of age on immunocompetence. The study found that inter-individual variability (57.9%; 37.2% young and old, respectively) was higher than the intra-individual (20%) variability. Our inter-individual variability prior to treatment for

the 38 participants was somewhat lower, 27.7%. Where we had data at all three doses for all three toxicants for the same individuals (n=16) the inter-individual variability was 27.4%. Froebel et al (1999) examining records for 10-42 months, from eight laboratories conducting 4,328 lymphocyte proliferation assays, found inter-individual variations in lymphocyte proliferation responses to mitogens to have a high coefficient of variation (CV) ~36-65%. Measuring proliferative capacity within individuals by looking at within-week variations for each of several stimulants (including PHA), Froebel observed greater variation between individuals than between different laboratories. The study concluded that at such high levels of variability there are very large differences in the levels of proliferative capacities between healthy individuals. While our number of study subjects are lower, our baseline variability and treatment variability ranged from (CV)~28%-43% this difference might be accounted for by the previously discussed larger number of study subjects (Froebel,1999) and operational and methodological differences discussed previously.

Epidemiologic Applications

With respect to the application of this assay in epidemiological studies, the differential responses as measured by cell proliferation were highly dependent on intrinsic proliferative responses. Intrinsic proliferative responses were determined by treatment with PHA but no toxicant treatment. For example lymphocytes from those individuals with a low intrinsic proliferative capacity were stimulated by treatment with B(a)P and DEE except at the highest dose. In contrast, those with a higher intrinsic proliferative capacity were suppressed by treatment with B(a)P and DEE. Thus unless there was a

possibility that one could obtain before and after exposure blood samples, one could not infer that the exposure to agents such as B(a)P and DEE was influencing proliferative capacity. Although a secondary goal of this research was to ascertain the intra-individual differences in intrinsic proliferative capacity by determining proliferative capacity in the presence and absence of toxicant, we were not able to recruit sufficient numbers of subjects within the time constraints of the study. Without this kind of information it is difficult to ascertain whether the variability would be related to the intra-individual effects or the treatment itself. Two epidemiological studies have attempted to use the LPA to measure effects of exposure on the immune system, Holland et al (2002) looking at pesticide applicators and Soto et al (2003), a measure of effects in children who had consumed arsenic in drinking water compared to controls. Because the dose of sodium arsenite we used in this study approximated blood levels found in the two epidemiological studies, we believe that this assay could be applied to epidemiological studies related to arsenic exposed individuals. Clearly the half life of the chemicals (B(a)P and DEE) would need to be assessed and careful selection of pre and post exposure measurements would need to be made. Because diesel exhaust extract is a complex mixture of PAHs and nitro-substituted PAHs, a molecular weight to calculate concentrations is not known. We used 252.3g/ mole, the molecular weight of B(a)P to represent the molecular weight of the DEE, but may have underestimated the approximate concentration of the PAH. Additional experiments are planned at higher doses of DEE given these factors.

One focus of this study was to evaluate intrinsic proliferative capacity as a biomarker of exposure for usage in field epidemiological studies. With high and low

responders showing differential effects, upon treatment with toxicants, careful planning for use in the field is required. With no clear indicators of prior exposures, investigators would need to select specific exposure scenarios where each person would serve as their own control and levels of exposures were such that very high concentrations of toxicants were expected, to utilize this assay. While no "true baseline" can be known for any individual, establishment of an acceptable intrinsic proliferative capacity for individuals would be necessary. Compounds that are eliminated rapidly from the circulating blood would provide optimal pre-exposure, post-exposure measures (clearance-baseline) exposure scenarios. A few examples are; seasonal exposure such as pesticide applications where before during and post- exposure measurements could be collected, occupational exposure followed by clearance on non-work days with similar pre and post collections.

In conclusion we have shown a great degree of variability between individuals' in vitro lymphocyte proliferative capacities both before and after exposure to three toxicants of environmental significance. The experimental design of this study allows for a relatively simple screening for purposes of assessing 1) the comparative immunotoxicity of chemicals, 2) identification of sensitive individuals or immnocompromised individuals and 3)a common mode of action between chemicals and within chemical types measured as immunotoxicity. Sodium arsenite was shown to be particularly suppressive to proliferation and cytotoxic compared to B(a)P and DEE. B(a)P and DEE have similar suppressive effects on proliferative capacity of in vitro T-lymphocytes. Of interest from an epidemiological point of view is that the concentrations in medium approximates concentrations in blood seen in environmentally exposed individuals. Our data suggests,

to be successfully applied in field studies, intrinsic proliferative capacity in the absence of environmental exposures would need to be established. Use of the lymphocyte proliferation assay in epidemiologic field studies can provide useful biomarker data, but the establishment of intrinsic data for the study subjects, and several repeat samples should be analyzed. Further experiments using higher doses and additional chemicals are planned and evaluations of intra-individual differences at baseline are needed.

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Appendices

Appendix A

Appendix A Illustration of 96 well plating schematic

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.75x10^{5}cells	0.75x10^{5}cells	0.75x10^{5}cells	0.75x10^{5}cells PHA 4.4µl	0.75x10^{5}cells PHA 4.4µl	0.75x10^{5}cells PHA 4.4µl	0.75x10^{5}cells +DMSO	0.75x10^{5}cells +DMSO	0.75x10^{5}cells +DMSO	0.75x10^{5}cells PHA 4.4µl +DMSO	0.75x10^{5}cells PHA 4.4µl +DMSO	0.75x10^{5}cells PHA 4.4µl +DMSO
B	BaP 0.2µM 0.75x10^{5}cells PHA 4.4µl	BaP 0.2µM 0.75x10^{5}cells PHA 4.4µl	BaP 0.2µM 0.75x10^{5}cells PHA 4.4µl	BaP 2.0µM 0.75x10^{5}cells PHA 4.4µl	BaP 2.0µM 0.75x10^{5}cells PHA 4.4µl	BaP 2.0µM 0.75x10^{5}cells PHA 4.4µl	BaP 20.0µM 0.75x10^{5}cells PHA 4.4µl	BaP 20.0µM 0.75x10^{5}cells PHA 4.4µl	BaP 20.0µM 0.75x10^{5}cells PHA 4.4µl	Media Blank	Media Blank	Media Blank
C	DE 0.2µM 0.75x10^{5}cells PHA 4.4µl	DE 0.2µM 0.75x10^{5}cells PHA 4.4µl	DE 0.2µM 0.75x10^{5}cells PHA 4.4µl	DE2.0µM 0.75x10^{5}cells PHA 4.4µl	DE 2.0µM 0.75x10^{5}cells PHA 4.4µl	DE 2.0µM 0.75x10^{5}cells PHA 4.4µl	DE 20.0µM 0.75x10^{5}cells PHA 4.4µl	DE 20.0µM 0.75x10^{5}cells PHA 4.4µl	DE 20.0µM 0.75x10^{5}cells PHA 4.4µl			
D	NaAs 0.02µM 0.75x10^{5}cells PHA 4.4µl	NaAs 0.02µM 0.75x10^{5}cells PHA 4.4µl	NaAs 0.02µM 0.75x10^{5}cells PHA 4.4µl	NaAs 0.2µM 0.75x10^{5}cells PHA 4.4µl	NaAs 0.2µM 0.75x10^{5}cells PHA 4.4µl	NaAs 0.2µM 0.75x10^{5}cells PHA 4.4µl	NaAs 2.0µM 0.75x10^{5}cells PHA 4.4µl	NaAs 2.0µM 0.75x10^{5}cells PHA 4.4µl	NaAs 2.0µM 0.75x10^{5}cells PHA 4.4µl			

Appendix A Dilution scheme for triplicate well analysis

Tube 1- 3.75 x 10^5 cells (375µl) + RPMI 125 µl

Tube 2- 3.75 x 10^5 cells (375µl) + PHA 22µl + RPMI 103 µl

Tube 3- 3.75 x 10^5 cells (375µl) + 1 µl DMSO Control Mix + RPMI 124

Tube 4- 3.75 x 10^5 cells (375µl) + PHA 22µl + 1 µl DMSO Control Mix + RPMI 102

Tube 5- 3.75 x 10^5 cells (375µl) + PHA 22µl + B(a)P 1 µl (0.0002M) + RPMI 102

Tube 6- 3.75 x 10^5 cells (375µl) + PHA 22µl + B(a)P 1 µl (0.002M) + RPMI 102

Tube 7- 3.75 x 10^5 cells (375µl) + PHA 22µl + B(a)P 1 µl (0.02M) + RPMI 102

Tube 8- 3.75 x 10^5 cells (375µl) + PHA 22µl + DE 1 µl (0.0002M) + RPMI 102

Tube 9- 3.75 x 10^5 cells (375µl) + PHA 22µl + DE 1 µl (0.002M) + RPMI 102

Tube 10- 3.75 x 10^5 cells (375µl) + PHA 22µl + DE 1 µl (0.02M) + RPMI 102

Tube 11- 3.75 x 10^5 cells (375µl) + PHA 22µl + NaAs 1.0 µl (0.0002M) + RPMI 102

Tube 12- 3.75 x 10^5 cells (375µl) + PHA 22µl + NaAs 1.0 µl (0.002M) + RPMI 102

Tube 13- 3.75 x 10^5 cells (375µl) + PHA 22µl + NaAs 1.0 µl (0.02M) + RPMI 102

Tube 14- RPMI 500 µl (place 100 µl in row B 10-12)

Appendix B

IRB: 02-EPA-201

Study I.D._____

Inter-Individual Variation Study: Sample Collection Data

Date: __/__/2003

Medical Station I.D. _____

Gender _____

Race _____

Height _____

Weight _____

Age _____

Sample Collection

Time of blood draw _____

Number of Tubes collected

Na Heparin (Green top) _____

Na Citrate (Blue top) _____

(Purple top) _____

Urine Sample collected _____**Yes** _____**No**

Nurse's Initials _____

Appendix C

EPA Human Studies Division
Chapel Hill, North Carolina

UNC-CH Study # 02-EPA-201

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

TITLE OF STUDY: *In vitro* cell model system to characterize inter-individual variation in sensitivity as measured by phenotypic responses to arsenic, diesel, and selected PM source-specific chemicals.

THIS CONSENT FORM SHOULD BE SIGNED ONLY BETWEEN 2/26/04 AND 2/26/05

APPROVED BY THE BIOMEDICAL IRB
THE UNIVERSITY OF NORTH CAROLINA

Principal Investigator: Scott Rhoney
Phone number: 919-966-9564
Consent Form Version Date: 11/17/03

Co-Investigators: Jane Gallagher, Ph.D., Reeder L. Sams II, Ph.D., Gary Hatch, Ph.D., Andrew Kligerman, Ph.D., Mike Schmitt, B.S., Judy Mumford, Ph.D, Mike Madden, Ph.D., Elizabeth Hilborn, DVM.

You are being asked to take part in a research study. The investigators listed above are in charge of the study; other professional persons may help them or act for them.

What are some general things you should know about research studies?
Research studies are designed to gain scientific knowledge that may help other people in the future. You may not receive any direct benefit from participating. There may also be risks associated with participating in research studies.

Your participation is voluntary. You may refuse to participate, or may withdraw your consent to participate in any study at any time, and for any reason, without jeopardizing your future care at this institution or your relationship with your doctor. If you are a patient with an illness, you do not have to participate in research in order to receive treatment.

Details about this particular study are discussed below. It is important that you understand this information so that you can decide in a free and informed manner whether you want to participate. You will be given a copy of this consent form. You are urged to ask the investigators named above, or staff members who may assist them, any questions you have about this study at any time.

What is the purpose of this study?
The purpose of this study is to obtain blood from study participants for the isolation of leukocytes to be used for laboratory exposure of these cell types to environmental pollutants to determine exposure-related effects on these cells. Also a urine sample will be collected to confirm that you are a non-smoker.

How many subjects will participate in this study?
If you decide to participate, you will be one of approximately 100 individuals in this research study.

How long will your participation last?
If you decide to participate, you will answer a few questions over the phone (~10 min.) to determine your eligibility for the study. You will then visit the EPA's Human Study Facility for a 1-hour appointment and at a later time for a 30-minute appointment.

Will you be paid for participating?
You will receive a maximum of $75 for your full participation in this study. Completion of the study will consist of 1) completion of a questionnaire, 2) donation of 2 blood samples, and 3) provide a urine sample.

ACTIVITY	PAYMENT
1st Blood donation	20
Urine sample	10
2nd Blood donation	20
Bonus for all three biological sample donations	25
TOTAL	**$75.00**

Will it cost you anything to participate?
There will be no costs to you for participating.

Who is sponsoring this study?
This research is funded by the U.S. Environmental Protection Agency.

What will happen if you are injured by this research?
In the event of personal injury resulting directly from the research procedures, financial compensation cannot be provided by the University of North Carolina at Chapel Hill nor can it be provided by the U.S.EPA under the Federal Employee Compensation Act. In spite of all precautions, you might develop medical complications from participating in this study. If such complications arise, the researchers will assist you in obtaining appropriate medical treatment but the University of North Carolina at Chapel Hill does not provide financial assistance for medical or other costs. You (or your insurer, Medicare, or Medicaid) will have to pay for any care that is needed. You do not waive any liability rights for personal injury by signing this form. In the event that physical injury is proximately caused by the negligence of a federal employee, the federal government would be liable in accord with the Federal Tort Claims Act (28 U.S.C. 2671-1680).

What if you want to stop before your part in the study is complete?
You can withdraw from this study at any time, without penalty. Your participation in this study is voluntary. The investigators also have the right to stop your participation at any time. Your participation may be stopped if you are determined to be ineligible for the study, or have failed to follow instructions, or because the entire study has been stopped.

What if you have questions about this study?
You have the right to ask, and have answered, any questions you may have about this research. If you have further questions, or if a research-related injury occurs, you should call Dr. Elston Seal at (919) 966-6217.

What if you have questions about your rights as a subject?
This research has been reviewed and approved by the Committee on the Protection of the Rights of Human Subjects (Medical IRB) at the University of North Carolina at Chapel Hill. If you have any questions or concerns regarding your rights as a research subject, you may contact the Chairman of the Committee at (919) 966-1344.

Subject's Agreement:

I have read the information provided above. I voluntarily agree to participate in this study.

Signature of Research Subject	Date
Printed Name of Research Subject	
Signature of Person Obtaining Consent	Date
Printed Name of Person Obtaining Consent	

THIS CONSENT FORM SHOULD BE SIGNED ONLY

BETWEEN 2/26/04 AND 2/26/05

Consent For Storing Blood, Tissue or Body Fluid
With Identifying Information

APPROVED BY THE BIOMEDICAL IRB,
THE UNIVERSITY OF NORTH CAROLINA

Addendum to Consent for Participation in:

IRB STUDY NUMBER 02-EPA-201, *In vitro* human cell model system to characterize inter-individual variation in sensitivity as measured by phenotypic responses to arsenic, diesel and selected PM source-specific chemicals.
PRINCIPAL INVESTIGATOR: Scott Rhoney

You are asked to give permission for some of your blood, tissue or body fluid (collectively referred to as "specimens") which will be collected in this research study to be stored for future medical research studies.

The specimens will be stored at the UNC School of Medicine, UNC Hospitals, or another site. All identifying information including your name and medical record number will be removed from the specimens and replaced with a code. Scott Rhoney and his/her associates will have access to the specimens and the code which links the specimen to you. There is no cost to you or your insurance company for the storage and use of the specimens.

Although every effort will be made to keep research records private, there may be times when federal or state law requires the disclosure of those records, including personal information about you. When disclosure is required, the UNC School of Medicine and/or UNC Hospitals will take all steps allowable by law to protect the privacy of your personal information.

By signing this form, you will donate the specimens for medical research purposes. Your donation does not entitle you to compensation from any commercial use of the products that may be derived from the specimen. The research studies in which the specimens may be used have not yet been determined, but they may involve genetic research. Before any research involving the specimens is conducted, a committee at the UNC School of Medicine called The Committee on the Protection of the Rights of Human Subjects will review and approve the research proposal.

In some cases, the Committee may require that you be contacted and asked for your consent to participate in the specific research study in which the specimens will be used. You have the right not to participate in any research study for which your consent is sought. Refusal to participate will not jeopardize your medical care or result in loss of benefits to which you are entitled.

In other cases, the Committee may require that you be notified about the results of a research study in which the specimens were used. You have the right to be told the results and their meaning, or to decide not to be told of those results, or to have the information sent directly to your personal physician.

You are asked to provide your social security number and agree that it may be used by

Scott Rhoney and his/her associates if it necessary to contact you to ask your consent to participate in a specific research study or to notify you about the results of the study.

The specimens may be shared with other institutions and research studies may be conducted at several locations at the same time. Non-identifying personal information about you will be provided to investigators from other institutions.

If in the future you should decide that you no longer wish for the specimens to be stored, you may contact Scott Rhoney and/or his/her associates at The University of North Carolina at Chapel Hill at (919) 966-9564 or The Committee on the Protection of the Rights of Human Subjects at (919) 966-1344 and request that the specimens be disposed of according to standard medical research procedures. If you do not make such a request, the specimens will be stored indefinitely. They may be disposed of at any time at the discretion of the investigators.

Before signing this consent form, please read the brochure entitled ***Information About Storage and Use of Specimens With Identifying Information*** that is designed to answer your questions.

- - - - - - - - - - - - -

Please check which course of action is to be followed in case, even with your social security number, the investigators cannot find you after reasonable time and effort:

_____I agree to allow the specimens to continue to be stored with identifying information, for as-yet-undesignated purposes that may include genetic research.

_____I request that the identifying code be removed from the specimens; after that is done, the specimens may continue to be stored and used for as-yet-undesignated purposes that may include genetic research.

_____I request that the identifying code be removed from the specimens; after that is done the specimens may continue to be stored and used for as-yet-undesignated purposes NOT INCLUDING genetic research.

_____I request that the specimens be disposed of.

I consent to the donation and storage of the specimens, as described above.

_____ _____

Name of Subject Date

_____

Social Security Number

UNC-CH SCHOOL OF MEDICINE / UNC HOSPITALS
INFORMATION ABOUT STORAGE AND USE OF SPECIMENS
WITH IDENTIFYING INFORMATION

This brochure provides information that may help you decide whether to allow some of your blood, tissue and/or body fluid (specimens) which will be collected as part of this research study to be stored and used for future medical research.

WHAT WILL HAPPEN TO THE SPECIMEN?

The specimens will be processed for storage, catalogued and placed in a secured facility at the UNC-CH School of Medicine, UNC Hospitals, or another site. All identifying information, including your name and medical record number, will be removed from the specimens. The specimens will be given a unique identifier (code).

The researcher in this study and his/her associates will have access to the specimens and the code which links the specimens to you.

WILL RESEARCH RECORDS AND PERSONAL INFORMATION BE KEPT PRIVATE?

Although every effort will be made to keep research records private, there may be times when federal or state law requires the disclosure of those records, including personal information about you. When disclosure is required, the UNC School of Medicine and/or UNC Hospitals will take all steps allowable by law to protect the privacy of your personal information.

IS THERE ANY COST FOR STORAGE OF THE SPECIMENS?

There is no cost to you or your insurance company for the storage and use of the specimens.

WHO OWNS THE SPECIMENS?

By signing the consent form, you will donate the specimens for medical research purposes. Your donation does not entitle you to compensation from any commercial use of the products that may be derived from the specimens.

HOW WILL THE SPECIMENS BE USED IN THE FUTURE?

The research studies in which the specimens may be used have not yet been determined. The studies may involve genetic research. Genetic research is about finding the specific location of genes, learning how genes work, and developing treatments and cures for diseases which are genetically based.

Before any research involving the specimens is conducted, a committee at the UNC School of Medicine called The Committee on the Protection of the Rights of Human Subjects will review and approve the research proposal. The Committee includes scientists and non-scientists, including community representatives. The purpose of the Committee is to assure that the interests of individuals participating in research studies are well protected.

WILL RESEARCHERS SEEK CONSENT TO DO FUTURE STUDIES INVOLVING THE SPECIMENS?

In some cases, the Committee may require that you be contacted and asked for your consent to participate in the specific research study in which the specimens will be used. You have the right not to participate in any research study for which your consent is sought. Refusal to participate will not jeopardize your medical care or result in loss of benefits to which you are entitled.

WILL YOU RECEIVE STUDY RESULTS OF RESEARCH INVOLVING YOUR SPECIMENS?

There may be times when the Committee will require that you be notified about the results of a research study in which your specimens were used. You have the right to be told of the results and their meaning, or to decide not to be told of those results, or to have the information sent directly to your personal physician.

HOW WILL RESEARCHERS FIND YOU IN THE FUTURE?

If you decide to allow the specimens to be stored and used in future medical research studies, you will be asked to provide your social security number. Your social security number will be used by the researchers and their associates in this study when it is necessary to contact you to seek your consent to participate in a specific research study or to notify you about the results of that study.

If you allow your specimens to be stored with identifying information, you will be asked to choose, at the time you sign the consent form, a course of action that will be taken in the event that the researchers are unable to locate you in the future, even with your social security number. The options include allowing continued storage and use of your specimens with the identifying code remaining, continued storage and use of the specimens after removing the identifying code, and disposing of the specimens according to standard medical procedures.

WILL THE SPECIMENS BE SHARED WITH OTHER INSTITUTIONS?

The specimens may be shared with researchers from other institutions. Research studies may be conducted at several locations at the same time.

No identifying personal information about you will be provided to researchers from other institutions who will use the specimens.

HOW LONG WILL THE SPECIMENS BE STORED?

The specimens will be stored indefinitely. Specimens may also be disposed of at any time at the discretion of the investigators, using standard medical procedures. If in the future you should decide that you no longer wish for the specimens to be stored, you may contact the researcher and/or his/her associates on the study in which you are participating. You may also contact The Committee on the Protection of the Rights of Human Subjects at (919) 966-1344 and request that the specimens be disposed of.

Appendix D

Inter-Individual Variation Study: Interview Questionnaire

Inter-individual Variation Study: Interview Questionnaire

1. What is your birth date? mm/dd/yyyy __/__/__
2. What is your sex? (Male or Female) _____

For the next 16 questions place a check mark in the box beside the answer that most accurately describes your activities or consumption or fill in the blank.

3. How often do you exercise (greater than 20 minutes)?

   Never ☐
   Occasionally (1-3 times/week) ☐
   Regularly 3 or more times/week) ☐

4. Do you take vitamins or dietary supplements?

   Never ☐
   Occasionally ☐
   Regularly ☐
   If so what kind? _____

5. How often do you eat fruit? (including fruit juices, for example orange juice)

   Less than 1 serving/day ☐
   1 serving/day ☐
   Greater than 1 serving/day ☐

Inter-Individual Variation Study: Interview Questionnaire

6. How often do you eat vegetables? (including vegetable juices, for example V-8)

- Less than 1 serving/day ☐
- 1 serving/day ☐
- Greater than 1 serving/day ☐

7. How often do you eat red meat?

- Less than 1 serving/day ☐
- 1 serving/day ☐
- Greater than 1 serving/day ☐

8. How often do you eat fish?

- Less than 1 serving/month ☐
- 1-5 servings/month ☐
- More than 5 servings/month ☐

9. How often do you consume alcoholic beverages?

- Never ☐
- Less than 1/day ☐
- 1 /day ☐
- Greater than 1 /day ☐

10. How often are you exposed to sunlight (greater than 30 minutes)?

- Never ☐
- Occasionally (1-3 times/week) ☐
- Regularly (greater than 3 times/week) ☐

Inter-Individual Variation Study: Interview Questionnaire

Seasonally (summer months, 1-3 times/week) ☐

11. How often do you visit a tanning salon?

Never ☐
Occasionally (1-3 times/month) ☐
Regularly (greater than 3 times/month) ☐
Seasonally (summer months, 1-3 times/week) ☐

12. Do you consider your overall stress level to be:

Mild ☐
Moderate ☐
High ☐

13. Do you have any existing medical conditions?
(for example: asthma, allergies, inflammatory diseases)

Yes ☐ No ☐

If yes, describe briefly_____

14. In the past month have you had any minor ailments? (For example, a cold, virus, significant cut or bruises)?

Yes ☐ No ☐

If yes, describe briefly_____

15. In the past 6 months have you participated in other research studies?

Yes ☐ No ☐

16. How often are you exposed to second hand smoke?

Inter-Individual Variation Study: Interview Questionnaire

Never	☐
Occasionally (1-3 times/week)	☐
Regularly (greater than 3 times/week)	☐

17. Since yesterday at this time (24-hour time period), what foods and beverages have you consumed?

_____
_____
_____
_____

18. Females Only- Are you currently pregnant?

Yes ☐ No ☐

Appendix E

Comparison of Doses	Control	B(a)P 0.2μM	B(a)P 2.0μM	B(a)P 20μM	DEE 0.2μM	DEE 2.0μM	DEE 20μM	$NaAsSO_2$ 0.02 μM	$NaAsSO_2$ 0.2 μM	$NaAsSO_2$ 2.0 μM
All (n=38) mean ± s.d. p value C.V.	102 ± 27.7 28	99.1 ± 23.5 0.196* 23.7	93.7 ± 23.4 0.017** 25	79.2 ± 26.4 0.002*** 33.3	102.6 ± 25.1 0.047* 24.5	103 ± 29.7 0.017** 28.8	84.7 ± 31.4 0.014*** 37.1	99.6 ± 23.8 0.3# 23.8	94 ± 23.8 $7x10^{-7}$## 25.3	79.2 ± 26.9 $1x10^{-6}$### 43.02
Group 1 (n=18) mean ± s.d. p value C.V.	123 ± 21 17.1	95.6 ± 15.6 0.2 * 18.2	89.2 ± 14.2 0.002** 15.2	69.6 ± 17.3 0.008 ** 24.4	99.8 ± 14 0.3* 14	95.9 ± 15.9 0.02** 16.5	80 ± 20.2 0.006*** 25.3	100.9 ± 26.1 0.13# 25.8	87.6 ± 21.9 $4x10^{-7}$## 25	36.4 ± 17.1 $1x10^{-5}$### 46.9
Group 2 (n=18) mean ± s.d. p value C.V.	81.2 ± 8.6 8.6	102.8 ± 27.4 0.31* 26.6	97.8 ± 28.5 0.15** 29	87.2 ± 29.7 0.07*** 34.1	109.6 ± 26.5 0.4* 24.2	112.8 ± 32.7 0.07 ** 29	93 ± 36.2 0.09*** 39	98.2 ± 17.6 0.36# 17.9	101.6 ± 31.4 0.005## 30.9	66.8 ± 22.1 $4x10^{-4}$### 33
Comparisons: B(a)P ,DEE; * 0.2μM to 2.0 μM; **2.0 μM to 20 μM; ***20 μM to 0.2 μM										
Complete Set (n=16) mean ± s.d. p value C.V.	96.3 ± 24 27.4	101 ± 28.1 0.4* 27	99.2 ± 28.2 0.1** 27.6	86.5 ± 29.5 0.08*** 33.1	106.7 ± 29.3 0.5* 26.6	106 ± 35.2 0.1** 32	91.7 ± 36.9 0.1*** 39	102.6 ± 25.9 0.4# 24	99.1 ± 36.8 0.0004## 36	57.9 ± 23.5 0.0003### 39

Group A (n=8)	117.9 ±	93.8 ±	90.5 ±	78.2 ±	99.4 ±	95.7 ±	79.5 ±	103 ±	95.6 ±	48.5 ±
mean ±	13.6	16	16	15	19.2	29.7	30.8	35.5	32.3	23.5
s.d.		0.3*	0.07**	0.03***	0.3*	0.1**	0.05***	0.4#	0.006##	0.04###
p value	11.5	17.2	17.6	19.2	19.3	31	38.7	34.5	33.8	47.5
C.V.										
Group B (n=8)										
mean ±	74.5 ±	108.1 ±	107.8 ±	94.9 ±	114.1 ±	117.7 ±	103.9 ±	102.2 ±	102.5 ±	67.4 ±
s.d.	12.3	36.2	35.8	38.5	36.7	38.8	40.3	15.6	42.8	21.2
p value		0.5*	0.2**	0.2***	0.4*	0.2**	0.3***	0.5#	0.014##	0.0006###
C.V.	16.6	33.5	33.2	40.6	32.2	32.9	38.8	15.2	41.8	31.5
Comparisons: Sodium Arsenite # 0.02μM to 0.2 μM; ## 0.2 μM to 2.0 μM; ### 20 μM to 0.02 μM										

Appendix F

National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 1975

Diesel Particulate Extract

This Standard Reference Material (SRM) is intended for use in evaluating analytical methods for the determination of selected polycyclic aromatic hydrocarbons (PAHs) in diesel particulate extracts and similar matrices. In addition to certified and reference values for selected polycyclic aromatic hydrocarbons, reference values are provided for the concentrations of selected nitro-substituted PAHs, extract residue mass, and mutagenic activity. All of the chemical constituents for which certified and reference values are provided in SRM 1975 were naturally present in the particulate material before extraction. A unit of SRM 1975 consists of four ampoules, each containing approximately 1.2 mL of a dichloromethane extract of diesel particulate matter collected from an industrial diesel-powered forklift.

Diesel particulate matter from the same lot of material that was used to prepare SRM 1975 is also available as SRM 2975, Diesel Particulate Matter (Industrial Forklift) [1].

Certified Concentration Values: Certified values for concentrations, expressed as mass fractions, for eight PAHs are provided in Table 1. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or accounted for by NIST. The certified values for the PAHs are based on the agreement of results obtained at NIST from two or more independent analytical techniques.

Reference Concentration Values: Reference values for concentrations, expressed as mass fractions, are provided for 23 additional PAHs (some in combination) in Table 2. Reference values for concentrations of 18 nitro-substituted and dinitro-substituted PAHs are provided in Table 3. A reference value for the extract residue mass is provided in Table 4. Reference values for mutagenicity in the Salmonella plate-incorporation assay are summarized in Table 5. Reference values are noncertified values that are the best estimate of the true value; however, the values do not meet the NIST criteria for certification and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods. Explanations in support of each reference value are given as notes in Tables 2 through 5.

Expiration of Certification: The certification of SRM 1975 is valid until **31 December 2007**, within the measurement uncertainties specified, provided the SRM is handled and stored in accordance with the instructions given in this certificate. However, the certification is nullified if the SRM is damaged, contaminated, or modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Return of the attached registration card will facilitate notification.

The coordination of the technical measurements leading to the certification of SRM 1975 was under the leadership of S.A. Wise of the NIST Analytical Chemistry Division.

The support aspects involved in the preparation, certification, and issuance of this SRM were coordinated through the NIST Standard Reference Materials Program by B.S. MacDonald.

Willie E. May, Chief
Analytical Chemistry Division

Gaithersburg, MD 20899
Certificate Issue Date: 19 December 2000
See Certificate Revision History on Last Page

Nancy M. Trahey, Chief
Standard Reference Materials Program

Consultation on the statistical design of the experimental work and evaluation of the data were provided by M.G. Vangel of the NIST Statistical Engineering Division.

The diesel particulate material was provided by M.E. Wright of the Donaldson Company, Inc., Minneapolis, MN. The extract was prepared by M.J. Hays of the NIST Analytical Chemistry Division.

Analytical measurements for the certification of SRM 1975 were performed at NIST by D. Bezabeh, M. Lopez de Alda, R. Deisenhofer, D.L. Poster, and L.C. Sander of the NIST Analytical Chemistry Division. Additional analytical measurements for the nitro-substituted PAHs were provided by M. Nishioka of Battelle, Columbus, OH and C. Chiu of the Analysis and Methods Division, Environment Technology Centre, Environment Canada, Ottawa, Canada. Mutagenicity data were provided by T.J. Hughes, J. Lewtas, and L.D. Claxton of the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC. The measurements of the extractable residue mass were provided by L. Gratz of Michigan Technological University, Houghton, MI.

NOTICE AND WARNING TO USERS

Storage: SRM 1975 must be stored in its original ampoule at temperatures less than 30 °C away from direct sunlight.

Handling: This material is an extract of naturally occurring diesel particulate material and contains constituents of known and unknown toxicities and mutagenicities. Therefore, appropriate caution and care should be exercised during its handling and use.

Instructions for Use: Samples of the SRM for analysis should be withdrawn from ampoules and used immediately. The certified values are not valid for materials in ampoules that have been stored after opening, even if resealed.

PREPARATION AND ANALYSIS[1]

Sample Collection and Preparation: The diesel particulate material was obtained from M.E. Wright of the Donaldson Company, Inc., Minneapolis, MN. The material was collected from a filtering system designed specifically for diesel-powered forklifts [2]. This diesel particulate material was selected based on recommendation by J. Lewtas, U.S. Environmental Protection Agency, Research Triangle Park, NC. The diesel particulate material was received at NIST in a 55-gal drum. The material was removed from the drum and homogenized in a V-blender for 1 h and then stored in polyethylene bags. A total of 13.7 kg of diesel particulate material was homogenized; a total of 5.65 kg of material was extracted for preparation of SRM 1975 and the remaining diesel particulate material was bottled for distribution as SRM 2975 [1]. Subsamples of approximately 38 g of diesel particulate material were extracted for 24 h with 800 mL of dichloromethane (DCM). Six subsamples were extracted per day for 25 days for a total of 5.6 kg of diesel particulate matter extracted. The extracts from each day were combined and concentrated by evaporation under nitrogen. During the 25 days of extracting the diesel particulate material, the concentrated extract was stored at 40 °C. The final combined extract solution was concentrated to approximately 8 L; this solution was then ampouled with 1.2 mL of extract per ampoule.

Polycyclic Aromatic Hydrocarbons (Tables 1 & 2): The general approach used for the value assignment of the PAHs in SRM 1975 was similar to that reported for the recent certification of several environmental matrix SRMs [3-6] and consisted of combining results from analyses using different chromatographic separation and detection techniques. This approach consisted of cleanup of the extracts using different solid phase extraction (SPE) or normal-phase liquid chromatography (LC), followed by analysis using the following techniques: (1) reversed-phase liquid chromatography with fluorescence detection (LC-FL) analysis of the total PAH fraction, (2) reversed-phase LC-FL analysis of isomeric PAH fractions isolated by normal-phase LC (i.e., multidimensional LC), (3) gas chromatography/mass spectrometry (GC/MS) analysis of the PAH fraction on two stationary phases of different selectivity, i.e., a 5 % (mole fraction) phenyl-substituted methylpolysiloxane phase and a smectic liquid crystalline stationary phase. The analysis of SRM 1975 has been described in more detail [5].

Two sets of GC/MS results, designated as GC/MS (I) and GC/MS (Sm), were obtained using two columns with different selectivities for the separation of PAHs. For GC/MS (I) analyses, duplicate subsamples approximately

[1] Certain commercial equipment, instrumentation, or materials are identified in this report to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by National Institute of Standards and Technology nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

0.8 g each from nine ampoules of SRM 1975 were analyzed. An internal standard solution (SRM 2269 and SRM 2270) of perdeuterated PAHs was added to the extract subsample, and then the extract was passed through a silica SPE cartridge and eluted with 2 % DCM in hexane. The processed extract was then analyzed by GC/MS using a 0.25 mm i.d. x 30 m fused silica capillary column with a 5 % phenyl-substituted methylpolysiloxane phase (0.25 μm film thickness) (DB-5 MS, J&W Scientific, Folsom, CA). For GC/MS (Sm) analyses, 0.8 g subsamples from six ampoules of SRM 1975 were prepared as described above for GC/MS (I) using an aminopropylsilane SPE cartridge. The processed extract was then analyzed by GC/MS using 0.2 mm i.d. x 25 m (0.15 μm film thickness) smectic liquid crystalline phase (SB-Smectic, Dionex, Lee Scientific Division, Salt Lake City, UT).

Two sets of LC-FL results, designated as LC-FL (Total) and LC-FL (Fraction), were used in the certification process. Subsamples of approximately 0.8 g from each of six ampoules of SRM 1975 were spiked with the perdeuterated internal standards (SRM 2269 and SRM 2270) and processed through two aminopropylsilane SPE cartridges connected in series to obtain the total PAH fraction. A second 0.8 g subsample from each of six ampoules was processed as described above and the total PAH fraction was then fractionated further on a semi-preparative aminopropylsilane column (μBondapak NH_2, 9 mm i.d. x 30 cm, Waters Associates, Milford, MA) to isolate a four to six aromatic ring PAH fraction as described previously [7-9]. The total PAH fraction and the four to six aromatic ring PAH fractions were analyzed using a 5-μm particle-size polymeric octadecylsilane (C_{18}) column (4.6 mm i.d. x 25 cm, Hypersil-PAH, Keystone Scientific, Inc., Bellefonte, PA) with wavelength programmed fluorescence detection [8,9].

Homogeneity Assessment for PAHs: The homogeneity of SRM 1975 was assessed by analyzing duplicate samples of 0.8 g each from nine bottles selected by stratified random sampling. Samples were processed, and analyzed as described above for GC/MS (I). No statistically significant differences among ampoules were observed for the PAHs at the 0.8 g sample amount.

Nitro-Substituted PAHs (Table 3): SRM 1975 was analyzed at NIST and two other laboratories for the determination of nitro-substituted PAHs. At NIST, four samples of SRM 1975 (~0.8 g each) were spiked with the following perdeuterated nitro-PAHs for use as internal standards: 9-nitroanthracence-d_9, 3-nitrofluoranthene-d_9, 1-nitropyrene-d_9, and 6-nitrochrysene-d_{11}. Each sample was divided into two subsamples which were then processed through an aminopropylsilane SPE cartridge using 40 mL of 20 % DCM in hexane. The concentrated eluant was then subjected to normal-phase LC using a semi-preparative amino/cyano phase column with a mobile phase of 20 % DCM in hexane to isolate the nitro-PAH fraction. The nitro-PAH fraction was analyzed by GC with negative chemical ionization mass spectrometry (GC/NCI-MS) using a 0.25 mm i.d. x 30 m fused silica capillary column containing a 5 % diphenyl-substituted dimethylsiloxane phase, 0.25 μm film thickness, Hewlett-Packard, Palo Alto, CA.

Additional nitro-PAH measurements were provided by Battelle, Columbus, OH and the Analysis and Methods Division of Environment Canada, Ottawa, Canada. For the Battelle measurements, five samples were prepared at each of two concentrations of extractable mass (0.2 and 2.0 mg/mL). Each sample was spiked with the following perdeuterated nitro-PAHs: 1-nitronaphthalene-d_7, 3-nitrobiphenyl-d_9, 4-nitrobiphenyl-d_9, 9-nitroanthracene-d_9, and 1-nitropyrene-d_9. Each sample was analyzed by GC/NCI-MS on a 60 m 5% phenyl-substituted methylpolysiloxane column (32 mm i.d., 0.25 μm film thickness). For the Environment Canada measurements, three samples were spiked with the following perdeuterated nitro-PAHs: 2-nitrofluorene-d_9, 9-nitroanthracene-d_9, 3-nitrofluoranthene-d_9, 1-nitropyrene-d_9, 6-nitrochrysene-d_{11}, and 6-nitrobenzo[*a*]pyrene-d_{11}. Each sample was analyzed by GC on a 30 m 5 % phenyl-substituted methylpolysiloxane column (25 mm i.d., 0.25 μm film thickness) with high resolution mass spectrometry detection using negative chemical ionization (GC/NCI-HRMS).

Extract Residue Mass (Table 4): The concentration of the extract residue mass was determined from triplicate measurements from each of six ampoules of SRM 1975. A 50 μL subsample of SRM 1975 was allowed to evaporate to dryness; after reaching constant mass, the residue mass was determined.

Mutagenicity Assay (Table 5): The values for the mutagenic activity of SRM 1975 were determined within an Environmental Protection Agency (EPA) laboratory using the *Salmonella typhimurium*/mammalian microsome mutagenicity assay [10-12]. The protocol for this study [13] was a modification of the protocol used in a previous 20 laboratory international study [14,15] on three other SRMs; SRM 1650 (a diesel particulate sample), SRM 1649 (an air particulate sample), and SRM 1597 (a coal tar extract). Modifications were made based upon the recommendations from the participants of the international study [16]. This study used tester strains YG1021 [17], YG1026 [17], TA98NR [18] and TA100NR [18] in addition to strains TA98 and TA100 that were used in the previous studies.

Prior to testing, an aliquot of SRM 1975 was solvent-exchanged into dimethylsulfoxide (DMSO) to create a starting concentration of 1.0 mg SRM 1975/mL DMSO (equivalent to 1 μg/μL). Adhering to published guidelines [19], the standard *Salmonella typhimurium* plate incorporation assay [10] was used. Modifications that developed from the recommendations noted above included the following: (1) the base layer agar contained trace amounts of histidine and biotin rather than being incorporated in the overlay agar, (2) the plates were incubated for 72 h, (3) the colony counter was calibrated before each use with both hand-counted plates and a template disk with a known number of dots which simulated colonies, (4) each test round had duplicate plates per dose and 10 arithmetically-spaced doses that were contained within one order of magnitude, and (5) four test rounds were conducted. The GeneTox Manager software [20] was used to record the data and to generate comparative slope values using three different statistical programs [21-23]. Table 5 provides the average slope values and the percent coefficient of variation for these values for the four experiments with each condition. A more thorough presentation of methods, results, and conclusions for the mutagenicity assay of SRM 1975 has been published [13].

Although the EPA laboratory providing the mutagenicity data may not be representative of all laboratories that conduct the Salmonella assay, this laboratory, which was established in 1980, has been involved in a variety of collaborative studies, and is well published in the use of the assay. Results, therefore, can be said to come from a well-established laboratory and are likely to represent "typical" bioassay results for SRM 1975.

Table 1. Certified Concentrations for Selected PAHs in SRM 1975

	Mass Fractions (mg/kg)[a]
Phenanthrene[b,c]	8.00 ± 0.20
Fluoranthene[b,c]	13.5 ± 0.6
Benz[*a*]anthracene[b,d,e]	0.092 ± 0.015
Chrysene[c,d,e]	1.95 ± 0.07
Triphenylene[c,d,e]	2.38 ± 0.10
Benzo[*b*]fluoranthene[c,d,e]	3.20 ± 0.10
Benzo[*k*]fluoranthene[c,d]	0.174 ± 0.050
Benzo[*e*]pyrene[b,d]	0.268 ± 0.023

[a] Each set of results is expressed as the certified value ∀ the expanded uncertainty. Each certified value is a mean of the means from two or more analytical methods. For results from two methods, the certified value is the equally weighted mean; for results from three or more methods, the certified value is the mean weighted as described in Paule and Mandel [24]. Each uncertainty, computed according to the CIPM approach as described in the ISO Guide [25], is an expanded uncertainty at the 95 % level of confidence, which includes random sources of uncertainty within each analytical method and among methods, as well as uncertainty due to the variation among the bottles. The expanded uncertainty defines a range of values within which the true value is believed to lie, at a level of confidence of approximately 95 %.

[b] GC/MS on 5 % phenyl-substituted methylpolysiloxane phase

[c] LC-FL of total PAH fraction

[d] GC/MS using a smectic liquid crystalline phase

[e] LC-FL of the four to six aromatic ring PAH fraction

The concentrations for selected PAHs in Table 2 are provided as reference values because either the results have not been confirmed by an independent analytical technique as required for certification or the agreement among results from multiple methods was insufficient for certification. Although bias has not been evaluated for the procedures used, the reference values should be useful for comparison with results obtained using similar procedures.

Table 2. Reference Concentrations for Selected PAHs in SRM 1975

	Mass Fractions (mg/kg)[a]
Naphthalene[b]	0.67 ± 0.01
1-Methylnaphthalene[b]	0.39 ± 0.01
2-Methylnaphthalene[b]	0.69 ± 0.02
Biphenyl[b]	0.24 ± 0.01
Fluorene[b]	0.110 ± 0.003
1-Methylphenanthrene[b]	0.50 ± 0.04
2-Methylphenanthrene[b]	1.7 ± 0.1
3-Methylphenanthrene[b]	0.88 ± 0.07
4- and 9-Methylphenanthrene[b]	0.25 ± 0.03
1,2-Dimethylphenanthrene[b]	0.017 ± 0.002
1,6-, 2,5-, and 2,9-Dimethylphenanthrene[b]	0.14 ± 0.01
1,7-Dimethylphenanthrene[b]	0.122 ± 0.008
1,8-Dimethylphenanthrene[b]	0.026 ± 0.003
2,6-Dimethylphenanthrene[b]	0.14 ± 0.01
2,7-Dimethylphenanthrene[b]	0.127 ± 0.008
3,5 and 3,6-Dimethylphenanthrene[b]	0.090 ± 0.009
Benzo[*ghi*]fluoranthene[c]	4.3 ± 0.1
Pyrene[b,d]	0.42 ± 0.13
1-, 3-, and 7-Methylfluoranthene[b]	0.092 ± 0.004
Benzo[*c*]phenanthrene[b,c]	0.51 ± 0.07
Benzo[*ghi*]perylene[b]	0.038 ± 0.006
Indeno[1,2,3-*cd*]pyrene[b]	0.12 ± 0.01
Dibenz[*a,c*]anthracene and Dibenz[*a,h*]anthracene[b]	0.079 ± 0.013

[a] Each set of results is expressed as the reference value ± the expanded uncertainty. Each reference value is the mean of the results from one analytical method or the equally weighted mean of the means of two analytical methods. Each uncertainty, computed according to the CIPM approach as described in the ISO Guide [25], is an expanded uncertainty at the 95 % level of confidence, which includes random sources of uncertainty within each analytical method and between methods. The expanded uncertainty defines a range that contains the best estimate of the true value, at a level of confidence of approximately 95 %.

[b] GC/MS on 5 % phenyl-substituted methylpolysiloxane phase

[c] GC/MS using a smectic liquid crystalline phase

[d] LC-FL of the four to six aromatic ring PAH fraction

The concentrations for selected nitro-substituted PAHs in Table 3 are provided as reference values because the results have not been confirmed by an independent analytical technique as required for certification. Although bias has not been evaluated for the procedures used, the reference values should be useful for comparison with results obtained using similar procedures.

Table 3. Reference Concentrations for Selected Nitro-substituted PAHs in SRM 1975

	Mass Fractions (mg/kg)[a]
1-Nitronaphthalene[b]	0.013 ± 0.003
2-Nitronaphthalene[b]	0.039 ± 0.006
4-Nitrobiphenyl[b]	0.05 ± 0.03
9-Nitroanthracene[c]	1.36 ± 0.03
9-Nitrophenanthrene[c]	0.27 ± 0.01
2-Nitrofluoranthene[b]	0.06 ± 0.03
3-Nitrofluoranthene[c]	1.47 ± 0.01
8-Nitrofluoranthene[b]	0.25 ± 0.03
1-Nitropyrene[c]	16.4 ± 0.2
4-Nitropyrene[b]	0.07 ± 0.01
7-Nitrobenz[*a*]anthracene[c]	1.62 ± 0.03
6-Nitrochrysene[c]	0.782 ± 0.007
6-Nitrobenzo[*a*]pyrene[c]	0.641 ± 0.006
1-Nitrobenzo[*e*]pyrene[c]	0.83 ± 0.05
3-Nitrobenzo[*e*]pyrene[c]	2.2 ± 0.2
1,3-Dinitropyrene[c]	0.60 ± 0.01
1,6-Dinitropyrene[c]	1.39 ± 0.04
1,8-Dinitropyrene[c]	1.55 ± 0.02

[a] Each set of results is expressed as the reference value ± the expanded uncertainty. Each uncertainty, computed according to the CIPM approach as described in the ISO Guide [25], is an expanded uncertainty at the 95 % level of confidence. The expanded uncertainty defines a range that contains the best estimate of the true value, at a level of confidence of approximately 95 %.

[b] Reference value is based on measurements made at Battelle.

[c] Reference value is based on measurements made at NIST. NIST values were confirmed by measurements at Battelle and/or Environment Canada; confirmation measurements were typically within 25 % of the NIST measurements.

The extract residue mass in Table 4 is provided as a reference value because the result is method specific as defined by the procedure described in the Preparation and Analysis section. Although bias has not been evaluated for the procedure used, the reference value should be useful for comparison with results obtained using a similar procedure.

Table 4. Reference Value for Extract Residue Mass in SRM 1975

Residue Mass	19.3 ± 0.2 mg/mL[a]

[a] This set of results is expressed as the reference value ± the expanded uncertainty. The reference value for the extract residue mass is the mean value of 18 measurements. Each uncertainty, computed according to the CIPM approach as described in the ISO Guide [25], is an expanded uncertainty at the 95 % level of confidence. The expanded uncertainty defines a range that contains the best estimate of the true value, at a level of confidence of approximately 95 %.

The mutagenicity results in Table 5 are provided as reference values because the results are method specific as defined by the corresponding procedures described in the Preparation and Analysis section. Although bias has not been evaluated for the procedures used, the reference values should be useful for comparison with results obtained using similar procedures.

Table 5. Reference Values for Mutagenicity (revertants/μg of organic extract) of SRM 1975[a]

Strain	Average Without S9 Activation	%CV	Average With S9 Activation	% CV
Results based on Bernstein et al. [21] model slope values				
TA98	462	8.6	125	13.7
TA100	68	16.8	65	15.2
TA98NR	158	37.8	50	48.5
TA100NR	45	6.5	18	18.7
YG1021	612	8.4	226	5.4
YG1026	137	18.5	121	8.7
Results based on Krewski et al. [22] model slope values				
TA98	465	3.7	121	14.5
TA100	111	28.2	65	16.8
TA98NR	181	42.4	44	44.8
TA100NR	46	9.1	18	18.9
YG1021	630	5.0	224	6.5
YG1026	158	17.4	126	8.8
Results based on Stead et al. [23] model slope values				
TA98	587	20.2	147	15.9
TA100	120	23.7	72	15.6
TA98NR	205	42.9	80	58.3
TA100NR	48	11.5	28	16.1
YG1021	672	11.8	257	9.5
YG1026	178	31.5	136	7.8

[a] A detailed description of the determination of the mutagenicity values for SRM 1975 is provided in Hughes et al. [13].

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Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Program at: (301) 975-6776, Fax (301) 926-4751, e-mail srminfo@nist.gov, or via the Internet http://www.nist.gov/srm.